

Is there an end to holy wars?

There are three lessons to be learned so far from the war in the Gulf, of which the chief is that an eventual settlement in the region will be reached with such difficulty that work on it had better begin now.

WHAT people have most generally learned from the past week's events in the Middle East is that the war that began last Wednesday is a high-technology war. The world's television networks have delighted in showing 'smart' bombs entering the doors of missile silos, or seeking out the air-conditioning shafts at the tops of high buildings. Taxpayers in the United States and Europe have marvelled that cruise missiles have been sighted making their way along Baghdad city streets towards precision targets down the road, and have been impressed that so much has changed since Vietnam. The Pentagon must no doubt be looking forward to the budget cycle about to begin again in Washington with unaccustomed equanimity.

Yet the Gulf War is not the high-technology war it might have been — and might have been a few years from now. The weapons systems now in service have been the best part of 15 years in development, and are the survivors of a much larger portfolio of weapons then commissioned. Moreover, to the military, they have obvious defects. The cruise missiles are subsonic, and can be chased and shot down by conventional aircraft; there is, as yet, nothing in service that will travel as quickly as the Tornado aircraft. Even the smart bombs require that human beings (aircraft pilots) should identify their targets; it will be some time before anybody realizes the military dream that each projectile will be provided with a neural network capable of recognizing its assigned target and no other. Data-acquisition is also an unsolved problem, to judge from accounts of how the records of reconnaissance aircraft must be reprocessed in desert laboratories before it can be told what they have seen.

Nor is it self-evident that these developments are beneficent. Their immediate advantage is that a single aircraft sortie can more certainly accomplish its objective, and probably cause more damage than would otherwise be the case. The lives of the pilots and their companions are therefore less at risk, which is, in itself, a laudable objective, but one that conceals dangers for the future. If it is ever possible to mount offensive battles without risking people's lives, the world will hardly be a safer place. Threats and counter-threats of automatic warfare will be no more comfortable than the promises of mutual destruction the superpowers have lavished on each other over the past 40 years.

Sadly, for the time being at least, the only restraint is the cost of automatic warfare. That is the second lesson so

far. Even the United States is not as lavishly equipped with advanced weapons as its generals would wish. Excited speculation about remaining Iraqi stocks of Scud missiles may yet be matched by brooding about US stocks of Tomahawk cruise missiles (believed to be about 500 before the war began), which cost about \$10 million each. The events of the past few months have shown that the United States is now the only superpower left on the surface of the Earth. The deployment in the Middle East is a straightforward manifestation of power, executed with much greater panache than, say, the Soviet deployment of force in Afghanistan in the 1980s. But even the remaining superpower has an Achilles' heel — the federal budget deficit to which the US Congress must bend its mind in the weeks ahead.

Wars, unfortunately, are distracting — dangerously so. The danger now is that, just as the US Congress will be tempted to hide from the budget deficit, so the rest of us will not pay sufficient attention to the problems of the Middle East, from which the war itself has sprung. There has been some talk of a 'new order' in the Middle East, but nobody knows what that is meant to be, while even the most limited interpretations of the phrase are mostly not feasible. Some, for example, would 'internationalize the oil'; with whose consent, and (more to the point) at what price? Some would press for arrangements by which 'all the people of the Middle East' would share in the wealth of this lucky natural resource, but there is no way in which such a utopian recipe can be foisted on sovereign states, which differ among themselves in their reserves and in their inclinations, and whose sense of sovereignty will only be strengthened if, in the end, Kuwait is wrested from Iraq.

The favourite remedy is that the war should be followed by a peace conference to resolve the issues raised by the Palestinian populations of the West Bank and of the Gaza strip; Western governments have taken to reiterating their commitment to this end for the past several years. Yet that project is doomed to failure without Israeli compliance, and would be counted a failure if its outcome did not include the recognition by the other states in the region of Israel's right to exist. That is but one unpalatable truth to swallow.

Even if that could be secured from the governments now in place (or intended to be restored), there can be no assurance that matters will remain like that. Indeed, the



yearning for peace and quiet might otherwise have persuaded many governments in the region to offer just such an assurance. But they are acutely aware that doing so would increase the internal pressure to which they are exposed from the fundamentalist Moslems their populations include. Even the schemes now in fashion for building regional security arrangements on foundations that would be acceptable in Central Europe are unlikely to succeed while the character of a government's relationships with its neighbours is determined by the changing balance of doctrinal opinion within its borders.

This is but another reason why the future of the Middle East must rest with the countries directly concerned. Certainly there is no immediate prospect that stability could be magicked out of the sand by persuading the countries of the region to become parliamentary democracies in the Western mould. Many of them are openly religious states, with the Koran (with all its freedom for alternative interpretations) standing for a Bill of Rights. So, too, are Iran and Pakistan. In circumstances like these, outsiders cannot simply say that the governments concerned should change their ways. The other side of that coin is that the burden must fall on those in the Middle East who have rightly feared bullying by Iraq (one of the most secular of Moslem states) to devise a framework for mutual relations likely to survive the strains ahead. Sadly, the Middle East may be long on oil reserves, but it is short on constructive argument. But unless it can find a prospectus for ending the holy wars that plague the region, this will not be the last high-technology war to afflict it. □

Farming fiasco

Europe's amended proposals on agriculture may unblock the GATT negotiations, but have no other merits.

LIFE goes on, perhaps most conspicuously at the European Commission, which has been struggling this week on behalf of the European Communities (EC) to present the General Agreement on Tariffs and Trade (GATT) with a more enticing proposal on agriculture than was rejected last month. If the howls of protest from European farmers' organizations are a reliable index, the EC might be thought to be pointing in the right direction. But it is not. The Commission's proposals, instead of helping to place European agriculture on a rational economic foundation, would perpetuate removeable anomalies indefinitely.

None of this implies that the Commission has an easy task. That the vice in which it is trapped is of Europe's own making is not much comfort. This year, it seems likely that the cost of meeting the EC's commitments to support the prices paid to farmers will increase by no less than 26 per cent, to a total of about \$40,000 million. (The costs of storing unwanted food, or of exporting it at knock-down prices, are extra.) But the essence of the change required to let the GATT negotiations resume is

that subsidies should be cut, and drastically. How to square that circle?

The Commission's first attempt is a recipe for winning the worst of all worlds. Support prices would be drastically reduced — by as much as 40 per cent in the case of cereals, for example — which would have the effect of making some export subsidies unnecessary. But it is also proposed that there would be arrangements to make sure that small farmers would be at least partly protected from the effects of these changes on their pocket-books. Farmers on a grander scale, on the other hand, would be paid for taking land out of production. One consequence of the proposed changes would be to replace direct price supports by compensatory payments of other kinds. Farmers of all sizes accurately guess that they would be worse off. But another consequence would be a general reduction of the efficiency of European farming.

The reasons why the EC consistently (and perversely) puts the cart before the horse on European farming derive from the Treaty of Rome, and from the belief which it enshrines that farmers should not be relatively impoverished by the great industrial resurgence then foreseen. In the event, agricultural technology has made the most dramatic advances in the past forty years, with the result that Europe is grossly over-farmed. Schemes for encouraging exotic crops will take up only a little of the slack. At some stage, Europe will have to bite the bullet of persuading farmers to do something else. Compensating them for the social upheaval entailed in doing something else would be proper. Paying them to be inefficient is, by comparison, foolish and probably self-defeating, for farmers are an ingenious lot. □

Inventive Britain

The British government's decision that the British Technology Group should be sold marks the end of an era.

THE British Technology Group (BTG) is the residuary legatee of two brave ideas, represented by the National Research Development Corporation and the Industrial Reconstruction Corporation (IRC). The first, created immediately after the Second World War, was meant to turn into commercial reality the supposedly wasted bright ideas of British researchers. It has done useful work in the sponsorship of bridge-building, the manufacture of antibiotics (cephalosporins) and even the construction of hovercraft. But, while often seeming to researchers to be a friendly uncle, it could not play that role to all would-be dependents and inevitably came to seem to be just another venture capitalist. IRC, invented by the second post-war Labour Government, was always more controversial. It was designed to be a coercive industrial marriage broker. In each case, the idea was that the government would put industry to rights. It says much for BTG that it has survived in a changed climate and yet remained saleable. □

DoE rallies to save US fusion research programme

■ Congressional cuts half restored

■ Watkins softening on tokamaks

Washington

SQUEEZED by congressional budget cuts and an erosion of political support, the US fusion research programme is being cut back to include only the most essential projects and those that have international support. Among other things, these developments could result in the layoff of hundreds of scientists and the abandonment of a potentially promising alternative source of energy.

The cuts have left a cloud over a programme that many still consider the best long-term alternative to fossil-fuel energy dependence and its troubles, from oil wars to global warming. But the pressure has also jolted federal officials into action to save the programme. When the need for a strong alternative-energy programme has never been more plain, Energy Secretary James Watkins has rallied to the defence of the fusion programme — a shift that promises that there may be brighter days ahead for thermonuclear fusion research in the United States.

Earlier this month, Watkins submitted a proposal to Congress to shift funds within the Department of Energy (DoE) to make up half of the \$50 million cut from the programme this year. In a letter to the chairman of the Senate Energy Committee, Watkins proposed an accounting device that would restore \$25 million to the budgets of several US fusion facilities. His objective is to maintain the level of support for mainstream tokamak reactors. Congress is expected to approve the request.

Although this rearrangement of funds would restore support for the core of the fusion programme to about last year's level, other projects will nevertheless have to be sacrificed, Watkins says. Among the five experimental projects DoE plans to phase out are two new projects — the Confinement Physics Research Facility at the Los Alamos National Laboratory and the Advanced Toroidal Facility at the Oak Ridge National Laboratory — designed to investigate the basic physics of plasma confinement.

Even with \$25 million restored, Watkins told Congress, "work on [the five] alternate concepts and advanced tokamak concepts . . . will still be stopped, prematurely narrowing the program to conventional tokamaks only".

As a consequence, Los Alamos announced last week that 65 employees of its fusion programme will lose their jobs. Oak Ridge is also expected to make substantial layoffs. The original \$50 million

cut would have cost between 600 and 750 jobs, only some of which can be saved by the proposed reaccounting, Watkins warned.

By streamlining the existing fusion programme, Watkins said he was trying to keep alive his plans for a new major fusion initiative. Last year he commissioned a panel known as the Fusion Policy Advisory Committee (FPAC) to recommend the future direction for DoE's fusion effort, much criticized by politicians as its optimistic promise seems always to be a constant 30 years from fulfilment.

FPAC panel recommended last year that DoE should proceed as soon as possible to the next big step in fusion research: a "burning plasma experiment". That is meant to be Princeton's planned Compact Ignition Tokamak (CIT), which would be the first machine to sustain a reaction that generates more energy than it consumes.

Once he has approval to restore the funds, Watkins is expected to endorse construction of CIT as part of a new "National Energy Strategy", itself an answer to critics who noted that the United States had been without a national energy plan for nearly two decades.

Watkins also intends to commit the United States to participation in the second phase of the International Thermonuclear Experimental Reactor (ITER), perhaps this week. In anticipation of the decision, DoE last week picked a consortium based around the University of California, San Diego, as its candidate for the ITER design headquarters. Japan and Germany (where ITER is currently

being designed) also have candidate sites for the second phase, a five-to-six year process that will produce an engineering design for the \$6,000-million international tokamak project. The four ITER partners (including the Soviet Union, which has not proposed a site) will meet next month in Vienna to begin the selection process.

Advocates of fusion see Watkins' moves as an encouraging signal in an otherwise sobered field. After two years in office, Watkins has yet to take a firm position on the DoE programme. As something of a fusion sceptic, he often recalls the rosy promises of cheap energy he heard in the 1950s when he was a young naval officer at Oak Ridge. Yet, when pressed by fusion advocates and environmentalists concerned over the decline in alternative energy research, Watkins assembled the FPAC and gave it free rein to recommend change (see *Nature* 347, 114; 1990). His letter to Congress appears to support the FPAC's ambitious recommendations. Before Congress slashed the programme's funds, Watkins said he had been prepared to issue a new fusion policy "based on the FPAC review and work by Department staff".

For the forthcoming 1992 budget, Watkins is said to have asked for \$360 million, an increase of more than \$30 million over DoE's request for this year. Although the White House, as part of a general austerity campaign, is expected to reduce that to about \$336 million when it releases the final budget request early next month, fusion advocates are encouraged by Watkins' apparent support. "We're hoping this shows that he strongly supports the program", says Steven Dean, head of industry association Fusion Power Associates. Just how much Watkins is prepared to do for the programme will soon become plain. As war rages over oil in the Middle East, the White House has asked DoE to release the new National Energy Strategy within weeks.

Christopher Anderson

ITALIAN RESEARCH

Three year plan to double science spending

London

THE Italian National Council for Science and Technology is due to meet research minister Antonio Ruberti this week to put flesh on the bones of a plan that aims to double in three years the proportion of Italy's wealth spent on civil research.

Ruberti's Ministry for Research, Universities and Technology and the advisory National Council for Science and Technology were both set up last year to focus Italian science policy and improve the country's research record. In 1988, Italy spent about 1.2 per cent of its gross domestic product (GDP) on civil research and development, compared with more

than 1.8 per cent in Britain and France and 2.7 per cent in West Germany.

Agostino LaBella, chairman of the Italian National Research Council's technology committee and a member of the National Council for Science and Technology, says doubling research spending in three years may be "wishful thinking", but four to five years is realistic — private sector spending is already set to meet this target, he says.

A full version of the plan will go to the Italian parliament later this year. LaBella expects research into telecommunications and transport to feature strongly.

Peter Aldhous

Späth loss rocks universities

Munich

GERMANY was rocked last week by the sudden resignation of Lothar Späth, the prime minister of the *Land* (state) of Baden-Württemberg, who was known during his 12 years in office as a champion of high-technology industry and of science and technology at universities. Under Späth's guidance, Baden-Württemberg became a base for modern industry that has in large part been responsible for the German economic strength of the past decade.

Späth resigned amid controversy after a series of damning articles appeared in the news magazine *Der Spiegel* alleging that he had taken a series of extravagant holidays in the Aegean and the Far East paid for by local industrialists, including executives of the electronics company SEL and the toothpaste company Blendax.

Späth denies that he was compromised by accepting the holidays. According to newspaper reports, he said in a hastily called press conference on 13 January that though there may have been "clumsiness in one or another case", he never lost his "inner independence" as prime minister. Before the scandal, he was thought to be one of the strongest competitors of Helmut Kohl for the office of national party chairman and a potentially strong candidate for chancellor.

Späth had come to see himself as the embodiment of Baden-Württemberg and the best proponent for its high-tech future, apparently blinding him to the appearance of impropriety in his actions. The news magazine *Stern* wrote last week that he "ran Baden-Württemberg like a well-oiled high-tech company".

Credited with almost single-handedly bringing to Baden-Württemberg such important institutions as the Centre for Molecular Biology in Heidelberg (ZMBH), Späth also brought supercomputers to universities in Stuttgart and Karlsruhe. No less important, Späth stood up for smaller projects in humanities such as a department of Japanese studies in Heidelberg, which he saw as essential to building stronger economic ties to Japan. "You could always count on Späth if you needed a few million quickly for a special project", says one university researcher.

Späth will be replaced in office by Erwin Teufel, who for the past 12 years has been head of the party caucus for Späth's party, the Christian Democratic Union, in the parliament of Baden-Württemberg. Teufel, 51 years old, served briefly as Interior Minister and Minister for Agriculture and Environment in the 1970s. Like Späth, he is a career politician with a degree from a polytechnic (*Fachhochschule*) in administration.

Researchers say that Späth's departure will have an unpredictable impact on science in Baden-Württemberg. Although it is hard to imagine anyone campaigning as hard for science and technology as Späth did, he neglected more mundane areas, such as general university budgets. Researchers hope Teufel will have a steadying influence on the government of Baden-Württemberg after Späth's mercurial management style.

For example, year after year for the past ten years, universities had inflicted on them a reduction in their budgets which, GULF CONFLAGRATION

though small in absolute terms (an average of DM2.5 million a year at a university the size of that in Heidelberg, which has an annual budget of roughly DM200 million), dramatically reduced the amount of money that could be freely invested by university administrators in additional research equipment and overhead costs.

In the interests of stability, Teufel is expected to take over the two-year budget plan for Baden-Württemberg that was about to be agreed before Späth resigned, according to CDU spokesman Gerhard Winter. He will then make changes, to be financed by supplementary budgets, as he sees fit.

Steven Dickman

Predicting climate problems

London

THE climate in the immediate region of Kuwait could be disrupted and the South-East Asian summer monsoon rainfall reduced if a large number of Kuwait's oil wells are set alight during the current hostilities, the UK Meteorological Office has concluded. But a global climatic disaster is unlikely, according to the preliminary analysis of the environmental effects of a conflagration, released by the British Ministry of Defence last week.

The Meteorological Office study was asked for by the British government after participants in a meeting in London predicted that sabotage of Kuwaiti oil wells could severely disrupt the regional, and even the global climate (see *Nature* 349, 96; 10 January 1991). It assumes that 80 million tonnes of Kuwaiti oil could burn over one year, if most wells are set alight.

Acid rain could be heavy in and around Kuwait, the Meteorological Office says,

but should be only a short-term problem.

The cloud of soot is the main uncertainty. Close to Kuwait, the blocking of sunlight is likely to cause "a considerable reduction" in daytime temperature, but the Meteorological Office predicts only a small effect globally. To depress the temperature in regions remote from Kuwait, the soot must first rise into the upper atmosphere. This depends on the size distribution of the soot particles and how quickly the soot is washed out of the atmosphere by rain, each of which is difficult to estimate.

But even a localized blocking of sunlight could reduce the heating that feeds the South-East Asian monsoon winds. Disruption of the monsoon raises the spectre of a drought in a highly populous region, but the Meteorological Office stresses that the monsoon system is very variable, making detailed predictions impossible.

Peter Aldhous

PRIZES

Chambon among winners of Swiss prize



Winners — Frank Grosveld (top left), Pierre Chambon (top right) and Hugh Pelham (left).

London

THE sixth Louis Jeantet Prize for Medicine, worth 1.95 million Swiss Francs, will be shared by three scientists: Pierre Chambon of the University of Strasbourg, Frank Grosveld of the National Institute for Medical Research in London and Hugh Pelham at the Medical Research Council Laboratory of Molecular Biology in Cambridge, United Kingdom. Chambon is recognized for work on gene expression, and is currently involved in the molecular biology of breast cancer. Grosveld, who is a Dutch citizen, is working on globin gene expression, whereas Pelham's group works on heat shock proteins and intracellular membrane traffic. All three researchers will use the prize, to be presented in Geneva on 19 April, to expand their research teams and buy new equipment.

Henry Gee

Money for space science

Tokyo

ASTRONOMY and space science have done particularly well in the 1991 budget for Japan's Ministry of Education, Science and Culture (MESC) which was released last week. Funds have been allocated to begin building one of the world's largest optical infrared telescopes in Hawaii and to develop a scientific satellite that will be sent to the Moon. Astrophysicists have also won some money to excavate a giant underground hole to accommodate the world's largest observatory for neutrinos and proton decay — provided that a budget for the observatory is allocated at some future date.

The highlight of the budget, which has to be approved by the Diet before it takes effect at the beginning of April but is unlikely to undergo change, is an allocation of ¥663 million (\$4.9 million) to begin construction of an 8-metre infrared optical telescope in Mauna Kea in Hawaii. Researchers at the National Astronomical Observatory, which until recently was part of Tokyo University, have been pushing for the telescope for years but have been hampered by MESC's limited budget and by bureaucratic regulations that make it difficult to establish facilities overseas (see *Nature* 311, 5; 1984).

Most of this year's allocation will go towards building the giant mirror for the telescope, says Makoto Kinoshita of

cost about ¥8,700 million (\$64 million), has yet to win full approval, it is most unlikely that anybody would dig such a big hole and not put anything in it.

Another project to win support is an ambitious plan from the Institute of Space and Astronautical Science (ISAS) to send a satellite to the Moon that will drop penetrators into the lunar surface to measure 'moonquakes'. The mission is a follow-up to ISAS's successful launch of a lunar probe last year (*Nature* 344, 693; 1990). An allocation of ¥380 million (\$2.8 million) has been made for the new lunar project in 1991. Apart from this project, the main reason for the large increase in MESC's allocation for space science is increased outlays for development of the M-V rocket that will carry the lunar spacecraft aloft and for a VLBI radiotelescope satellite that will be launched before the lunar mission.

MESC's science budget is subject to strict limitations laid down by the Ministry

of Finance and cuts have had to be made elsewhere to make way for the increased outlays for space science and astronomy. Among the projects to suffer is TRISTAN, the huge electron-positron collider at the High Energy Physics Laboratory in Tsukuba. Operating hours for the collider have been reduced by 100 hours to 2,800 hours in 1991. But MESC officials say they are still committed to the accelerator (there have been suggestions that TRISTAN's main ring should be converted into a 10-GeV synchrotron). Among the new items in MESC's budget is reference to a "new" and "large-scale" human genome project. But MESC officials say that the precise budget for the project has yet to be decided. Last year it was announced that the ministry hopes to increase grants for genome research from ¥280 million to about ¥500 million (\$3.7 million) per year in 1991. And MESC is also seeking ¥850 million over five years to establish a new Genome Analysis Centre at Tokyo University's Institute of Medical Science.

David Swinbanks

GENETIC ENGINEERING

TNF production licensed

Munich

In an encouraging development for the German pharmaceutical industry, the city of Ludwigshafen in southwest Germany last week approved the production of recombinant tumour necrosis factor (TNF) by the chemical and pharmaceutical company BASF AG.

This is only the fourth licence for the production of a recombinant medicine to have been issued in Germany. The others went to Boehringer Ingelheim for tissue plasminogen activator (TPA), Grünenthal AG for the anti-clotting drug Saruplase and Behringwerke AG for erythropoietin (EPO).

The approval was particularly encouraging for the biotechnology industry because it means that another region of Germany has decided that it can live with the risks of pharmaceutical production using genetic engineering. The situation is likely to improve, because future licence applications will be regulated under last year's federal law, which allows companies to win a licence without public participation if they use safety strains of bacteria.

BASF hopes to produce 500 grams of TNF a year for use against tumour-related ascitis (also known as abdominal dropsy), a condition of some abdominal cancers in which the abdominal cavity may accumulate as much as 10 litres of fluid. Hitherto, this condition has been relieved by withdrawing the fluid mechanically, but it quickly returned. Three treatments with TNF alleviate the condition, says a

BASF spokeswoman, although the tumours remain.

Despite early setbacks in the use of TNF to reduce tumours — it seemed to shrink all else but the tumours in early US trials — BASF remains hopeful that TNF will be useful in the treatment of cancer. In combination with α -interferon, TNF has been shown in clinical trials to help to shrink tumours and reduce metastases in 40 per cent of renal cell carcinoma patients.

The decision to grant BASF a licence may help to stem the exodus of pharmaceutically inspired genetic engineering research from Germany. In 1988, BASF decided to move most of its research in the field to the United States, which seemed to offer a friendlier climate than Germany.

There also seems to have been a regulatory impulse behind the application for a licence. BASF had produced the TNF used in German clinical trials at its home base of Ludwigshafen — the US Food and Drug Administration requires that a drug approved for clinical use must be produced in the same plant as that used for making the samples used in clinical trials.

BASF also applied last month to the Federal Health Office (BGA) in Berlin for permission to market TNF in Germany. If BGA finds the application in order, it will pass it on to Brussels, which can approve TNF for use in all 12 member states of the European Communities.

Steven Dickman

MESC BUDGET

	¥ Thousand million	percentage change from 1991
Grants-in-aid of research	58.9	+ 5.5
Government/industry research	13.4	+ 16.5
Donations from industry	44.3	+ 13.0
Nuclear fusion	8.8	- 1.8
Accelerator physics (TRISTAN)	15.8	- 2.7
Space science	20.8	+ 15.6
Astronomy	1.7	+ 95.2
Earth science	2.3	+ 1.1
Antarctic research	3.1	- 40.2
Domestic research fellowships	2.4	+ 8.5

MESC's research institute division. Several foreign companies are keen to get the job. A contractor will be selected after passage of the budget by the Diet, Kinoshita says.

Construction of the complete facility is expected to take eight years at a total cost of about ¥38,000 million (\$281 million).

Competing for funds is a proposal to build a giant observatory for neutrinos and proton decay in a mine in Gifu Prefecture. Researchers at Tokyo University's Institute for Cosmic Ray Research had hoped to get a budget for the observatory, called Superkamiokande, this year but instead will have to make do with funds to excavate a hole 40 metres wide by 40 metres deep for the facility. But MESC officials say that although Superkamiokande, which is expected to

A cleanish bill of health

London

THE system of 'peer review', used to judge research proposals, is showing "signs of strain" as competition for research council grants becomes ever more fierce, but there is "no practicable alternative". That is the sober conclusion of a working group commissioned by the Advisory Board for the Research Councils (ABRC) to examine ways in which the UK research councils' funding decisions could be streamlined and improved.

The group took evidence from more than 150 researchers, and was chaired by Margaret Boden, an expert in artificial intelligence from the University of Sussex and a former ABRC member. Most scientists believe that peer review — where research proposals are judged by the applicants' peers in the scientific community — worked reasonably well until the 1980s. But the past decade has seen a growing number of projects rated highly but still refused funding, as the number of applications for research council grants has risen by more than 15 per cent and the average value of such grants has more than doubled. Not surprisingly, researchers refused funding for alpha-rated projects have begun to question the system.

Quantitative measures of researchers' past output have been put forward as an alternative to peer review. But Boden's group decided that past performance cannot be used as the sole criterion to judge future research promise. In any case,

BRITISH TECHNOLOGY GROUP

measures such as citation counts may be unreliable — failing to account for variation in publication and citation practices between different disciplines and to distinguish between positive and negative citations. Nevertheless, the report says that the research councils could experiment with the use of citation indices to supplement peer review.

Boden's group also looked at the possibility of holding in-depth interviews with research grant applicants — a technique used by Venture Research International, a small organization set up to fund innovative and unorthodox research. The report says there is some merit in this approach, but the cost is likely to be prohibitive for the research councils, given the huge numbers of grant applications they must process each year.

Despite reaching the depressing conclusion that peer review is inherently fallible, the Boden report suggests a number of improvements to ease the current problems. Disgruntled researchers refused funding will be pleased with the recommendation that more feedback should be given to unsuccessful grant applicants, following the lead of the Economic and Social Research Council, which sends copies of referees' reports to all applicants. The research councils should also publish statements explaining their peer-review systems, the report says.

Boden's group was surprised to find that not all of the research councils have a

computerized database to keep track of referees used in peer review. The research councils should all establish databases on active researchers and their suitability as referees, the report concludes. These should be compatible, so the research councils can tap in to one another's resources.

The peer-review system has been accused of discriminating against unorthodox proposals and young researchers with no proven track record. The Boden group found no hard evidence of this, but suggests that money could be earmarked for young researchers and novel projects.

Peter Aldhous

ANIMAL EXPERIMENTS

UK government funds humane research

London

THE British Home Office has announced that an extra £145,000 will be spent this year on research to develop alternatives to animal experiments (see Correspondence page 274). The new money supplements an existing scheme worth about £70,000 a year, and £30,000 in outside donations administered by the Home Office.

Michael Balls, from the Fund for the Replacement of Animals in Medical Experiments (FRAME), welcomes the new money, but says it is "trivial" in the context of government expenditure. The government has yet to demonstrate "a genuine commitment" to reducing animal experiments, he says. FRAME receives about £250,000 a year from industry, charitable trusts and the European Communities.

Peter Aldhous

PRIZES

Japan Prize winners

Tokyo

THIS year's winners of the lucrative Japan Prize — Japan's answer to the Nobel Prize — are Jacques-Louis Lions, chairman of analysis and systems control at the Collège de France and president of the National Centre for Space Studies (CNES), France, and John Julian Wild, director of the Medico-Technological Research Institute of Minneapolis in the United States. Each will receive a cash prize of ¥50 million (\$370,000).

Lions, who is 62, is given the award by the Science and Technology Foundation of Japan in this year's category of applied mathematics for his pioneering research in the field of analysis and control of distributed systems and for his contributions to development of applied analysis. Wild, 72, receives his award in "imaging techniques in medicine" for his development of ultrasound imaging. The awards will be presented in Tokyo in April.

David Swinbanks

Privatization ahead for BTG

London

A MOVE to privatize the British Technology Group (BTG), the state-owned patents licensing company, was welcomed by the organization last week. On 17 January, Peter Lilley, UK Secretary of State for Trade and Industry, tabled the bill that would, if it became law, turn what has been a government agency into a private company.

With 1989-90 revenues of £29.5 million and pre-tax profits of £9.5 million, BTG claims to be the largest technology transfer licensing organization in the world. Privatization will enable BTG to adopt a profile less centred on the United Kingdom. The purpose of BTG is to act as a broker between inventors and the commercial world. In return for 50 per cent of profits, BTG takes on all the costs of patent application, some research and development funding, licensing an invention to industry and any necessary legal action.

This last service is particularly impressive, according to Bob Whelan of

the Centre for the Exploitation of Science and Technology, a UK body established just under three years ago to identify scientific developments with possible industrial application. "They've not been afraid to take on the heavyweights like the Pentagon — and beat them", he says. This refers to a case in which the US Department of Defense reached a \$6.1 million out-of-court settlement with BTG over the right to use a design of hovercraft skirt.

Jeremy Bray, who speaks for the Labour opposition on science, sympathizes with BTG's wish to go it alone. But he thinks that it will still enjoy something of a monopoly. "That they are only able to pass on 50 per cent of earnings to inventors suggests high costs", he says: "a competitor might do better". Whereas one source at the Department of Trade and Industry admitted that BTG has "the lion's share of the business", Whelan is emphatic that BTG will soon meet more competition once its activities are expanded abroad to the United States and Japan.

Henry Gee

Compromise eludes EC

Munich

To the dismay of consumers in Europe and developing countries, the European Communities (EC) have failed once again to gain Community-wide approval for food irradiation. Although irradiation has been approved in seven member states (most recently in Britain) and two others are in the process of approval, it is opposed by Germany and some others.

Although it is accepted that irradiation can prolong the shelf-life of foodstuffs by killing what bacteria they contain, the general application of the technique remains controversial. Some hold that irradiation may create potentially toxic chemicals, but governments such as that of Germany believe that irradiation is in any case unnecessary.

Italy, which held the EC presidency in the second half of 1990, had vowed to find a compromise, but the last EC Council meeting of the year in Rome failed to reach a conclusion.

An official of the Geneva-based World Health Organisation (WHO), Valery Abramov, says that WHO regards irradiation as a way of improving the food supplies of developing countries, and has therefore urged all countries to approve the procedure.

EC Commission officials in Brussels are also disappointed that no solution could be reached at Rome. "We see it as our role to protect the consumer", says one official. By allowing irradiation accompanied by proper labelling of irradiated foods, the EC could offer more variety to European consumers and "increase the trust for these products" in developing countries, which are often dependent on exporters for ensuring the quality of food products.

The negotiations appear to have broken down over the labelling of food products containing irradiated ingredients. Current EC regulations require labelling of ingredients that make up 25 per cent or more of the content of a foodstuff offered for sale. Most members would have accepted a similar threshold, but Italy and Spain insisted at Rome that irradiated ingredients should be labelled if they made up as little as "one-half of one per cent" of a product, says the official.

Germany and Luxembourg oppose all irradiation. The German Bundestag even voted in 1989 to try to persuade other EC member states to ban irradiation. But EC officials say it would probably be in Germany's interests to agree to common rules. Otherwise, Germany has no legal means of preventing the import of irradiated foods from other EC countries. It is widely believed that such imports have already begun.

Most of the present national laws would allow irradiation of any food product as

long as it has been scientifically proved to be safe. France, for example, permits irradiation of potatoes, onions, garlic and shallots (to prevent sprouting), and spices, cereals, poultry and frogs' legs (to reduce bacterial contamination).

The European Commission is frustrated by the dispute, fearing that it may end up before the European Court of Justice. Precedent suggests that EC members would have to accept irradiated food products from abroad provided they are safe and properly labelled.

Reflecting popular sentiments, the European Parliament recommended in 1989 that irradiation should be banned in Europe except for herbs and spices. These have usually been preserved using ethylene oxide, which was recently banned because of its carcinogenic properties.

In Germany, where irradiation of food products for the domestic market is banned without special permission, nobody has dared to apply for such approval. Food irradiation plants in former East Germany were closed by the federal government immediately after reunification. But Germany continues to export irradiated foodstuffs.

Opponents of food irradiation, mostly independent lobby groups, have shifted their strategy over the past ten years. At first, they contended that radiation products linger in irradiated food that may ultimately be harmful. But even the hardliners, frustrated by a lack of scientific evidence for their claims, are falling back on the argument that irradiation is not necessary and that consumers should not be forced to accept it.

In an official statement in 1987, WHO declared that irradiation is "harmless" in doses less than 10,000 Gray*. Normally, much smaller doses are used: less than 1,000 Gray to prevent sprouting in onions and potatoes or to kill insects. Larger amounts are necessary to eliminate bacteria.

Just 500,000 tonnes of food are irradiated annually, including 400,000 tonnes of Soviet wheat, according to a Bundestag report. And even in the Netherlands, where irradiation has been approved for several classes of foodstuffs, the amount consumed is equivalent to "less than one meal per person a year" according to the EC official. Both the high cost of irradiation facilities and the lack of consumer acceptance have limited the use of the technique.

Faced with stalemate, the Commission has been seeking a least-common-denominator regulatory solution. It tried to obtain a majority of member states to

agree to the irradiation of just two or three foods, and then to build up the EC-approved list slowly as member states approved irradiation. But this approach foundered both on the length of the list and on the labelling issue.

The Commission may now be forced simply to agree to license the irradiation process and to devise acceptable labelling regulations. One difficulty is that Luxembourg, which holds the presidency of EC for the first half of this year, is unlikely to hurry a solution.

That is how the issue may end up in the courts. As soon as an EC-based producer of irradiated food tries to export to Germany, says Professor Johannes Diehl of the Federal Institute for Nutrition at Karlsruhe, there is the possibility of a lawsuit, a turn of events that Diehl considers "very probable". And, just as in the case where (then West) Germany tried to keep out certain imported beers, "Germany will lose another one", he predicts.

The position of developing countries may eventually be influenced by the decision, last May, of the US Food and Drug Administration (FDA) that irradiation may be used to kill salmonella and other harmful bacteria in domestic or imported poultry. Although the US poultry industry is believed to have made little use of the technique, the decision has been seen as a "green light" by many developing countries, according to Abramov of WHO. He expects Latin American countries that export poultry to benefit economically from the decision.

Steven Dickman

AIDS RESEARCH

Better late than never for minorities

Washington

THE US Public Health Service, which has come under fire for neglecting minorities in its AIDS research, has decided to make amends. As part of a new programme, federal health agencies will spend more than \$6.2 million this year to include more minorities and minority institutions in AIDS research. Over \$2.4 million of the total will go to three institutions — Howard University, the University of Hawaii and the University of Puerto Rico — to pay for new equipment and personnel to bring them up to the research standards of an AIDS clinical trial centre. Much of the remaining funds will support the first clinical trials aimed specifically at minorities, who now make up some 44 per cent of all US AIDS cases.

Beyond increasing the representation of minorities in general clinical trials, the funding will support research on special risk factors for minority patients, including intravenous drug use.

Christopher Anderson

*One Gray of radiation, which is equivalent to 100 rads, corresponds to one joule of energy absorbed by one kilogram of material.

Animal procedures research

SIR—I am writing in my capacity as chairman of the research subcommittee of the Animal Procedures Committee about the new Home Office scheme to support research that could lead to a reduction, refinement or replacement of the use of living animals for experimental or other scientific purposes. The Animal Procedures Committee advises the Home Office on the priorities for such research and assists in the selection and evaluation of proposals.

The use of living animals in scientific procedures is an emotive issue that raises a number of ethical questions. There is considerable public awareness and concern about the purposes of many procedures and how they are carried out, which is often heightened by misleading reporting. However, the fact remains that regrettably the use of animals continues to be needed in many areas of research work and safety testing. It is of course incumbent upon those working in biomedical research to ensure that they use animals only where no suitable alternative exists, that they use the minimum number of animals necessary and that no avoidable

suffering is caused. These principles are enshrined in the Animals (Scientific Procedures) Act 1986, which imposes strict controls on the use of living animals in scientific procedures.

Much work is already being done by charitable bodies and commercial concerns to find alternatives to the use of living animals. But it is important that those involved in research work and safety testing are not only involved but are seen to be involved in seeking alternatives.

I am pleased to announce therefore that limited funds will be available from April 1991 for research in the United Kingdom into the reduction, refinement or replacement of the use of living animals in scientific procedures. The Animal Procedures Committee will give preference to research proposals that have a good prospect of leading to the refinement or replacement of procedures which use large numbers of animals or which involve substantial suffering, for example challenge tests and vaccine production. The committee also continues to be interested in the possibility of developing better measures of disease, discomfort and stress in labora-

tory animals, and in improvements in the husbandry of such animals. Grants will normally be awarded for periods up to three years.

Details of the research scheme and how to apply may be obtained from Mr Peter Edmundson, E Division, Room 971, Home Office, 50 Queen Anne's Gate, London SW1H 9AT — (071 273 2029). Completed applications will be considered in the first half of April this year.

ANDREW HUXLEY

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German education

SIR—Steven Dickman is correct to claim (*Nature* 348, 187; 1990) that university entrance standards in West Germany were generally relaxed in the 1960s, enabling more than a quarter of the age group concerned to attend university. This process has been welcomed by some and criticized by many others. But the assertion that East Germany selected its students much more strictly needs clarification.

In both parts of Germany, the gateway to university is the *Abitur* encompassing a number of oral and written examinations. In West Germany, any high-school student meeting the necessary academic requirements was granted the opportunity to reach his goal, but pupils in East Germany were selected by criteria only partly connected with their personal scholarly abilities. In fact, these criteria included socialist achievements as well as the political and social status of the pupil's parents, pupils from working-class families being preferred to those from parents with an academic background. As the headmaster of a school could make the final decision as to which of the pupils could continue towards the *Abitur*, many were not given this opportunity because of personal dislike, even though they met the necessary academic requirements. Others passed with comparatively low qualifications, as the quality of the teaching staff was measured by the number of pass grades among their own students.

Being myself a future student of medicine, it is painful to be once again faced with the myth of the superior East German student. In contrast, many school-leavers in East Germany were forced to do a manual job rather than being given the opportunity to study at university.

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Fraudulent slur

SIR—"Scientific fraud" has for some time been debated in scientific journals and the popular press on both sides of the Atlantic, usually in a cynical tone. Individual suspected cases have added grist to the mill^{1,2}. There is throughout an unstated but persistent assumption that individuals accused of scientific fraud are indeed guilty. There is something perverse in human nature that makes people pleased when respectable, prominent individuals are accused of wrongdoing. When this happens, the idea of giving someone the benefit of the doubt seems to disappear, as well as the concept of "innocent until proven guilty".

If anomalous data are found in the report(s) in question, error may have occurred in the typing/reviewing/editing process, instruments may have lacked calibration or been misread, a mistake made in cataloguing samples, or there may be misperception due to mental set. Individual lives may suffer as a result of mistakenly attributing fraud to a particular study.

I myself did a study as a graduate student that contained a number of errors³. They were due not to fraud but to sloppy editing/paperwork on my part. I have not been accused of scientific fraud, probably because of the study's insignificance.

Essentially, it was a simple study in social psychology. A car was parked by the side of the road between towns, with

the hood up to signify car trouble. A man or a woman would stand by the car and the number of vehicles stopping to offer assistance was tabulated. Simple. To no one's surprise, the overwhelming offers of assistance were offered to the woman.

The dumb mistakes in the paper occurred in citing references in the text that were forgotten in the reference section, and, when in calculating the χ^2 , the wrong column in the significance table was read, so that instead of $P \leq 0.001$ it was in reality $P \leq 0.01$.

Ironically, the one detail in the paper that looked suspicious *did* indeed occur. Over several hours, exactly the same number of other vehicles passed by the car for each participant.

Now, I can't help wondering if some of the serious accusations of scientific fraud bandied about are not due to similar embarrassingly simple, yet stupid mistakes. In one recent case involving AIDS research, in fact, the accused individual said the resulting uproar was "due to sloppy paper-writing in a tense period" (of competition)⁴. At the same time, it is also true that some other anomalous reports have been widely acknowledged to be due to "honest mistakes", and not to fraud⁵.

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Letters submitted for Correspondence should be typed, double-spaced, on one side of the paper only. □

Gene therapy in perspective

D. J. Weatherall

With the successes of molecular medicine and the recent announcement of a human gene transfer protocol, the medical world is asking whether gene therapists will now deliver the goods.

THE recent successes of molecular medicine must have gone at least some way to mollify those who believed that this field, while a useful form of occupational therapy for scientists, would have no practical value for patient care^{1,2}. Within a year of the first demonstration by molecular hybridization of a human gene deletion the same approach was used to identify a genetic disease in fetal life. Since then the molecular pathology of many single-gene disorders has been characterized, reverse genetics has identified the products of the genes involved in common disorders such as muscular dystrophy and cystic fibrosis, some of the multiple mutations that seem to underlie common cancers have been defined, and recombinant DNA technology has produced several agents of potential or already proven clinical value.

Throughout this period there has been a lot of activity directed towards correcting genetic disease by 'gene therapy'. A new journal devoted entirely to this topic has appeared³, and the field has attracted more than its share of media attention. Not surprisingly therefore, the medical world is starting to wonder when the gene therapists will deliver the goods. The recent publication of the protocol for a human gene transfer attempt which was submitted to the Human Gene Therapy Subcommittee of the National Institutes of Health, and news of its acceptance⁴, suggests that genuine progress has been made at last.

Why is it taking so long to correct a genetic disease in this way? Is it simply that the technology is still not fully developed, or have clinicians been slow in appreciating the potential of this new field for their patients.

Ethics

There are two main approaches to gene therapy. First, genes may be inserted into somatic cells, that is any body cell other than a germ cell. This raises no major ethical issues because it is essentially the same as organ transplantation; any changes in the genotype should be confined to the particular cell population in the recipient. The second, germ-line therapy, involves the introduction of genes into fertilized eggs. In this case the genes will be distributed in both somatic and germ cells and hence will be passed on to future generations, an outcome which would take us into completely new ethical territory.

Before patients are subjected to gene therapy, appropriate genes must be isolated and their regulatory regions defined. It is then necessary to identify and collect appropriate target cells and to develop safe and efficient vectors with which to introduce foreign genes. Finally, there must be unequivocal evidence from animal experiments that the inserted gene functions adequately, that the recipient cell population has a reasonably long life-span, and that the 'foreign' gene produces no deleterious effects.

There are several routes which somatic gene therapy might follow. The first, replacement therapy, is directed at the correction of recessive disorders due to the defective production of an enzyme or other protein. Here, one or more copies of the normal gene would be inserted into a cell to produce sufficient amounts of the missing gene product to correct the disorder or at least convert it into a heterozygous phenotype. Another attractive approach, 'correction therapy', will certainly be required for the management of dominant disorders associated with the production of an abnormal protein. This would use nature's way of gene swapping — a normal gene would be lined up with the defective gene allowing a recombination event between the two genes to correct the defect. Although considerable progress has been made towards developing site-directed recombination of this type^{5,6}, this approach is still far too inefficient to be contemplated for clinical use. Although attempts have been made to introduce genes directly by physical methods this route is also highly inefficient and, currently, the most promising approach involves the use of retroviral vectors⁷⁻¹⁰.

The use of retroviruses as vehicles for gene transfer requires a source of recombinant infectious viruses which are able to express the transferred gene after integration. It turns out that the viral proteins which comprise nearly 80 per cent of the retroviral genome can be deleted and replaced by DNA sequences encoding the gene to be transferred. Cells containing such a recombinant retrovirus are not able to produce infectious particles unless the structural viral proteins are supplied from elsewhere. This problem has been tackled in two ways¹⁰. First, the recombinant virus has been introduced into a cell line along with a wild-type retrovirus. These cells produce both replication-defective and

-competent virus with the potential disadvantage of a persistent retroviraemia. Second, the replication-defective virus can be introduced into a cell line already containing a retrovirus genome lacking the packaging sequence. The recombinant retrovirus can be packaged using structural proteins from the modified (helper) virus but is itself capable of only one further round of infection.

Difficulties

Initially the development of packaging lines ran into difficulties due to recombination between the recombinant and helper viruses¹⁰. These problems have been overcome by the development of lines which contain two helper viruses with different regions deleted, thus requiring at least two recombination events to produce active helper virus contamination. In this way marker genes have been transfected into haemopoietic stem cells — from which blood cells are derived — which have then been used to reconstitute lethally irradiated mice. The results suggest that self-renewing and pluripotent stem cells can be infected⁹.

Bone marrow cells from mice, primates and humans have been transfected with a variety of retroviral vectors containing genes such as hypoxanthine phosphoribosyl transferase (HPRT), purine nucleoside phosphorylase (PNP), adenosine deaminase (ADA), β -globin and several haemopoietic growth factors. The results of these experiments have been encouraging. But the selection of retroviral producer clones on the basis of expression in murine fibroblast cell lines such as NIH3T3 does not correlate with what happens in primary haemopoietic cells. Using a variety of promoters for the transfected genes, including SV40, thymidine kinase and metallothionein, it has been found that although excellent expression can be obtained in fibroblasts or haemopoietic cell lines there is only a very low level of expression in primary murine haemopoietic cells.

Problems have also been encountered in obtaining long-term expression of genes introduced into haemopoietic stem cells. Expression has usually been at a low level, in a minority of cell populations, and short-lived. A 'waxing and waning' phenomenon has been observed suggesting that only a small number of stem cells have been transfected and that haemopoiesis, at least in the mouse, may reflect the

sequential activation of different stem-cell clones. It is not yet known whether this is relevant to human stem cells. The pluripotent stem cell makes up only a small proportion of bone-marrow cells. Furthermore, for successful retroviral transfection these cells must be in cycle; it appears that the bulk of haemopoietic stem cells are out of cycle at any particular time. This suggests that the short-lived expression of transfected genes may simply reflect the fact that the bulk of cells that are transfected are more mature and hence have a limited lifespan. Some improvement has resulted from the addition of haemopoietic growth factors such as IL1, IL3 and IL6 during infection, and a variety of approaches to select for transfected cells have also been explored. But despite all this work it is still not absolutely clear why some transfected stem cells express 'foreign genes' and others do not.

Because of these difficulties it seemed likely that the first attempts at gene therapy would be restricted to 'house-keeping genes' such as ADA which are expressed in most cells at a low level and which do not require tight regulation. But the recent discovery of major regulatory elements which direct high-level tissue-specific expression of the globin genes^{11,12} has again raised the possibility of trying to correct the thalassaemias.

These advances, together with recent progress in the purification of haemopoietic stem cells and in the isolation of haemopoietic growth factors that are specific for early progenitors^{13,14} suggest that many of these problems should be surmountable in the next few years. Another recent success has been the retroviral transfer of genes into other cell types including keratinocytes, hepatocytes, endothelial cells and fibroblasts (see ref. 7). The latter are particularly attractive candidates because they are easily accessible and can be reimplanted in autologous skin grafts.

Progress

It appears therefore that genuine progress is being made towards the development of somatic-cell gene therapy, albeit limited for the time being to disorders that are due to defective function of genes in the progeny of haemopoietic stem cells. The fact that many of these conditions have been corrected by bone-marrow transplantation¹⁵ suggests that this approach will be feasible although it should be remembered that transplant patients are heavily immune-suppressed. Very little is known about whether the products of a transfected gene to which the recipient has had no previous exposure will mount an immune response. Data derived from patients with haemophilia and Christmas disease who have had long-term replacement therapy with the clotting factors VIII and IX suggest that this may well be the

case (E. Tuddenham, personal communication). It is possible, therefore, that recipients of foreign genes will have to receive prolonged immune-suppression treatment.

How can patients be selected for the first attempts at this completely novel form of treatment? Any completely new and potentially harmful form of treatment must be compared with alternative therapies. And here there is a problem. Those disorders which are expressed in the progeny of haemopoietic stem cells are, by and large, conditions for which there are other forms of treatment. Bone-marrow transplantation is remarkably successful in the genetic immune-deficiency disorders¹⁴, though the intense immune suppression required if marrow is transplanted from a non-HLA-matching sibling remains a serious problem; presumably this is the main argument for the gene transfer experiment which is now underway at NIH (ref. 4). The β thalassaemias can be managed by blood replacement and the judicious use of agents for the removal of iron, and up to 80 per cent of cases can now expect a good result after bone-marrow transplantation provided there is a suitable donor¹⁶, statistically, one in four siblings. Thus gene therapy will by no means revolutionize the management of these conditions. Much will depend on its efficiency, cost and level of discomfort to patients; bone marrow transplantation is expensive and is still dogged by serious complications.

So far the major role of recombinant DNA technology in clinical genetics has been for carrier detection and prenatal diagnosis of genetic disease. For disorders like the thalassaemias, where major programmes of this type have developed in tandem with adequate public education, there has been a remarkable reduction in the births of the severely affected homozygotes¹⁷; the same approach is being followed for other serious genetic diseases. But children with genetic diseases will continue to be born and hence work directed towards gene therapy should continue. It is, however, important to put this field into perspective. Because of the success of prenatal diagnosis programmes and of supportive therapy together with marrow transplantation, the impact of somatic gene therapy for disorders expressed in the progeny of haemopoietic stem cells may be quite small. There seems little doubt, however, that it will be feasible. Indeed it has already been done in an attempt to mark tumour-infiltrating lymphocytes¹⁸.

Assuming, therefore, that these early attempts at gene replacement are successful, and turn out to have no deleterious effects, what of the longer-term future for this field? Its role in the management of distressing multi-system single-gene disorders is still far from clear. The correc-

tion of these conditions may well entail genetic manipulation at a very early stage of development and we will undoubtedly require much more information about the pathophysiology of these conditions before tackling them in this way. But, as pointed out recently¹⁹, gene transfer has much broader potentials for the management of human disease than simply the correction of single-gene disorders. These include the insertion of genes for anti-clotting agents into the endothelial lining of vascular grafts, the insertion of genes for the LDL receptor into liver cells to attempt to reduce cholesterol levels in patients with hypercholesterolaemia, the targeting of anti-cancer cytokines by the integration of their genes into TIL cells, and the development of other sophisticated drug delivery systems. Many other ingenious ideas along similar lines are being aired, including the use of genetically engineered respiratory-tract viruses to be used in aerosols for the management of cystic fibrosis.

Although such speculations are the fuel which drives a complex field like gene therapy, our top priority must be to determine whether it is possible to correct a simple monogenic disease with complete safety. So far this has not been achieved in animal studies. For this reason there must be some concern about proceeding directly to gene transfer experiments in man. Given that there are other therapeutic options for managing many of the diseases that are the result of abnormal gene function in the progeny of haemopoietic stem cells, and the disastrous effects on this field that would follow from a major unforeseen complication, it is vitally important that the groups who are developing gene therapy continue to concentrate on trying to provide clear evidence of its efficacy and safety in animal models. □

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Another Landau sum made good

The long-standing problem of hydrogen atoms in a magnetic field seems to have been remarkably made plain by a distinctive collaboration between theoreticians and experimentalists.

ONE of the oddest features of the natural world we live in is that some of the simplest problems cannot be simply solved. The three-body problem is the most familiar: there is no closed expression for the motion of three bodies in gravitational interaction with each other except for special cases. And the device of supposing that the mass of one of the three is much smaller than those of its partners is no solution, but merely a means of making the smallest into a test-particle whose trajectory plots the known gravitational field of the other two.

It is almost as surprising, a good sixty years after the late Lev Landau looked at the problem, that there is so little that can be simply said about the behaviour of the simplest atom, that of hydrogen, when immersed in a uniform magnetic field. The question is that of what happens to the motion of the single electron in these circumstances. (Supposing the proton at the nucleus to be infinitely massive may simplify the algebra, but does not rid the problem of its complexity.) The surprise is that there is little that is simple that can be said, but the reason is more readily understood.

The simply soluble problems of quantum mechanics are those in which (if necessary, by a suitable transformation of coordinates) Schrödinger's equation becomes a hierarchy of three equations for each particle, each of which relates a momentum to functions of the corresponding (or conjugate, in the hamiltonian sense) space coordinate and to no others. Plainly, the presence of a significant magnetic field must undermine easy expectations of this kind, for the force (or rate of change of momentum) on a moving electron is perpendicular both to the field and to the direction of motion. That is why, as physicists would discover, the equations are not separable or, as mathematicians would prefer to say, the differential equation is not integrable.

It is not surprising that the practitioners should have a sense of being challenged by this state of affairs, but that does not explain why the problem of a hydrogen atom in a magnetic field has become as fashionable as in the past few years. Part of the explanation is that, especially when the electron is mostly a long way from the nucleus (as in an excited or 'Rydberg' state), its behaviour may be described as a delicate mixture of classical and quantum behaviour.

Still more compelling, it has also become clear that even the classical problem (with point-like electrons) behaves chaotically when the electron is only loosely bound. The explanation is that such an electron will move in orbits enormously stretched-out in the direction of the magnetic field, so that small differences of starting-conditions will be enormously magnified as time goes on. Who can resist the study of a system in which the correspondence of chaos in the classical version of a problem is mirrored by the complexity of the quantum version of the problem?

Even so, there is more than that to admire in two articles now in last week's issue of *Physical Review Letters* (14 January) in which a group from Paris first calculates the properties expected in a magnetic field from hydrogen atoms just below and just above the ionization threshold and then, together with a group from MIT, describes and interprets the results of measurements on the same system.

The theoreticians are D. Delande, A. Bommier and J. C. Gay, from the Ecole Normale Supérieure (66, 141; 1991). The experimentalists are Chun-ho Iu, George R. Welch, Michael M. Kash and Daniel Kleppner (66, 145; 1991). There is no internal evidence to show whether one must admire the theoreticians for their restraint in publishing only when the experiments were complete or the experimentalists for taking on trust the theoreticians' promise that their calculations would be feasible. Either way, the agreement between calculation and experiment is remarkable.

So are the experiments themselves. The MIT group has worked with lithium-7 rather than with hydrogen (which, at least for highly excited or unbound states, should make very little difference to the interactions). The trick is to use a superconducting solenoid to generate a field of 6 tesla (6,000 gauss) at which, the calculations show, interesting effects should become apparent. By generating an atomic beam along the axis of the solenoid and with the help of a little laser spectroscopy (with resolution of 10^{-3} cm^{-1}), the outcome is essentially an energy spectrum of the system looking for all the world like some spikey noise record. But it is not noise, for the measured spectrum agrees in an uncanny way with the calculated spectrum.

This, broadly, is what one would expect from Landau's work all those years ago. Precise calculations such as those now described were beyond the facilities at his disposal, but the physics of a complicated problem are often most apparent from the crudest approximations. And the most drastic approximation to the behaviour of a hydrogen electron is that in which the atomic nucleus is ignored, reducing the problem to that of the motion of a free electron in a magnetic field.

Unsurprisingly, all possible values of the energy are possible, but Landau's treatment made plain the contribution to the total energy of kinetic energy representing motion along the direction of the field (which is not quantized in this version of the problem) and that at right angles to it (which is quantized in units of $\hbar H/mc$, where H is the magnetic field and the other quantities refer to the mass and charge of the electron, Planck's constant and the velocity of light in the usual way).

These are still known as the 'Landau levels'. The next approximation to a true atom is then merely to impose the condition that the electron is geometrically confined, when the uncertainty principle can be used to select from among the otherwise multiply infinite array of Landau levels. And, after that, further refinement is possible by taking the electrostatic interaction between the proton and the electron as a perturbation.

Plainly the difficulty for a hydrogen atom near the ionization threshold is that, even in the absence of a magnetic field, there is an infinite number of energy levels, each differing from the previous level by a decreasing amount of energy, but corresponding to a very different and more extended orbit, beyond which there must lie a continuum of accessible states. So how does that system in a free atom mate with the system of energy-levels for a free electron in a magnetic field?

The calculators have set about their task with rigour, even zeal. At one stage they mention, almost in passing, that their approximation to the wave function of an unbound electron consists of an expansion involving 90,000 suitably chosen basis functions. At another, they note laconically that calculating rather less than ten per cent of the spectrum which is their main result occupied a Cray-2 machine for 2,000 seconds — rather more computer power than a PC buff could expect to use up in a lifetime.

John Maddox

The true turtles' story. . .

Nicholas Fraser

ALTHOUGH biologists are fully aware of what characterizes a turtle, the evolutionary history of the group is disputed — just as the tale the mock turtle tells in *Alice in Wonderland* is obscure and incomplete, so we are unsure whether certain fossils are mock ancestors or true ancestors. On page 324 of this issue¹, Reisz and Laurin take a step towards resolving the ancestry of the true turtles (Testudines), suggesting that they are the closest sister-group of the procolophonids, a group of rather poorly known Permian and Triassic tetrapods. That seemingly modest conclusion has implications that extend to the group Reptilia as a whole.

In recent years, ideas about the relationships of modern reptiles to one another have undergone a radical revision. Indeed, the great revolution in vertebrate palaeontology brought about by the introduction of cladistic classification raised the spectre of redundancy for the Reptilia; almost overnight the very essence of the group became threatened. The two homeothermic amniote groups, the birds and the mammals, retained their integrity, and at least the evolutionary relationships of certain fossil reptiles retained their links with these two groups. The mammal-like

reptiles were still recognized as members of the Synapsida, a monophyletic group which also includes the true mammals^{2,3}. Likewise, the association of the birds with the archosaurs (the so-called 'ruling reptiles' that include dinosaurs, crocodiles and pterosaurs) was maintained. But it became apparent that many of those animals traditionally regarded as reptiles, such as the lizards, crocodiles, snakes and turtles, did not seem to share any common character⁴. They instead became a disparate assemblage of amniotes that possessed neither feathers nor fur.

Somewhat paradoxically, what had threatened to bring about the demise of the reptiles as a natural group then came, at least partially, to its rescue. Cladistic analyses of living reptiles indicated that crocodiles, lizards, snakes and the tuatara are part of a monophyletic group called the Diapsida. The unique feature of the diapsids is the presence of an opening high up in the temporal region of the skull (Fig. 1). Some Palaeozoic fossils and many Mesozoic groups, including the Dinosauria and the Pterosauria, are also unquestionably diapsids, and many other extinct groups, including the plesiosaurs⁴ and the ichthyosaurs, were probably

derived from within the Diapsida. Furthermore a group of Palaeozoic tetrapods, the Captorhinidae, although lacking any openings in the temporal region (anapsid), are currently thought by many authors to be the sister group of the Diapsida^{5,6}.

Testudines, like the captorhinids, are anapsids. Although the anapsid condition is primitive for amniotes, in some cladistic analyses a handful of additional characters have been cited linking the Testudines and the captorhinids⁷⁻⁸. Consequently the Testudines and captorhinids have been brought together with the diapsids to restore the Reptilia⁹, albeit in a somewhat modified form. At the same time the synapsids are generally regarded as the sister group to the Reptilia (Fig. 2a). An alternative classification which regards the Testudines as the sister group of a combined Synapsida and Diapsida (Fig. 2b) has received less support. Both hypotheses offer a broad resolution of the interrelationships of the basal amniote

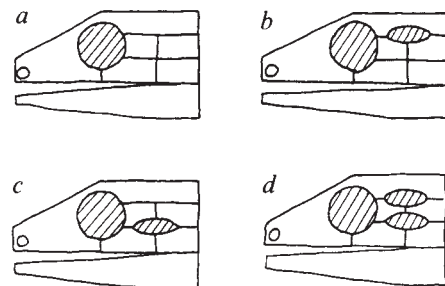


FIG. 1 Configurations of the temporal openings in the skull of a variety of reptilian groups. a, The anapsid skull which is the primitive reptilian condition. b, A single upper temporal opening which is the key feature of the diapsids, although diapsids generally also have a second opening (d) which may be modified, as in the lizards, to form a large embayment in the ventral margin of the skull. c, A single opening in the lower part of the skull is typically found in the synapsid lineage.

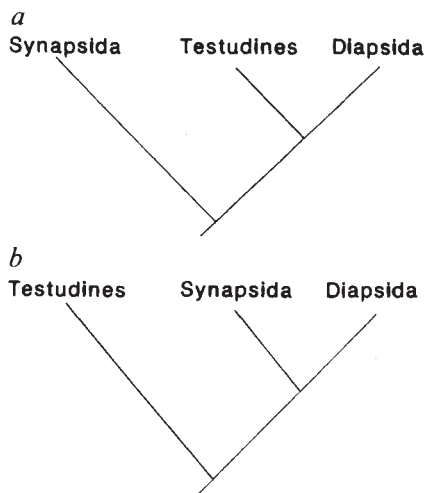


FIG. 2 Two alternative hypotheses for the evolutionary relationships of the main 'reptilian' taxa. a, The mammals and mammal-like reptiles (Synapsida) form the sister group to all those living animals traditionally regarded as reptiles. b, In this hypothesis the living reptiles are split into two quite separate groups.

taxa, but neither satisfactorily incorporates certain lesser known fossil taxa into the scheme. These poorly known taxa, which have been referred to as 'para-reptiles', include the pareiasaurs, the millerettids and, significantly, the procolophonids.

The new evidence described by Reisz and Laurin¹ comes from specimens of *Owenetta*, a fossil procolophonid found in South Africa. The authors list nine well-defined characters of the skull, and one of the postcranial skeleton, which are unique to procolophonids and Testudines. The similar configuration of the bones surrounding the ear region in both groups is particularly striking. The upshot is that the relationship between the Testudines and the captorhinids is not particularly well supported, thus throwing the constitution of the Reptilia into the melting pot once again. Separating the Testudines



"When we were little," the Mock Turtle went on at last, more calmly, though still sobbing a little now and then, "we went to school in the sea. The master was an old Turtle — we used to call him Tortoise —".

"Why did you call him Tortoise, if he wasn't one?" Alice asked. "We called him Tortoise because he taught us," said the Mock Turtle angrily. "Really you are very dull!"

from other extant reptiles places the spotlight on resolving the relationships of the 'parareptiles'. Further examination of these early amniote fossils is likely to prove fruitful in the quest to determine the evolutionary transition from an amphibian lifestyle to an entirely terrestrial one. The key to resolving whether the reptiles — and perhaps even the amniotes — are diphyletic clearly lies with our reaching a much better understanding of the procolophonids and of the other 'parareptilian' groups.

In the same way that Lewis Carroll's mock turtle is a concoction of two different animal groups, it might be that the reptiles will also ultimately prove to be a mixture of two quite separate tetrapod lineages. Indeed it is not inconceivable that the turtles will be shown to be more closely related to the synapsids than to

other living reptiles, thereby lending some credence to the mammalian features of the fictional mock turtle. □

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ATMOSPHERIC CHEMISTRY

Stratospheric ozone depletion over the Arctic

Martyn Chipperfield

It is now beyond doubt that stratospheric ozone is being destroyed by chlorine derived from man-made chlorofluorocarbons (CFCs). The largest decrease occurs over the Antarctic during the southern spring¹, where effectively all of the ozone is destroyed at certain altitudes in the lower stratosphere². But no such large ozone loss due to stratospheric chlorine has been unequivocally observed over the North Pole, partly because the meteorology of the Arctic is quite different³. On page 300 of this issue⁴, Hofmann and Deshler describe measurements of ozone in the Arctic lower stratosphere during January and February 1990 which seem to show that there is depletion. Moreover, the authors find that, given the presence of some sunlight, the degree of ozone loss observed is correlated with the amount of time air parcels spend in regions that are cold enough for the catalytically crucial polar stratospheric clouds to form.

Emerging picture

Although many quantitative details of the Antarctic ozone depletion remain unexplained, a broadly consistent picture has emerged⁵. During the austral winter a strong circumpolar westerly vortex is set up in the lower stratosphere. Inside this vortex the stratosphere cools allowing the formation of polar stratospheric clouds (PSCs). These clouds can be of two types. The first to form (type I) at around 195 K is believed to be composed of nitric acid trihydrate⁶. At colder temperatures water ice (type II) particles can form. These PSC particles perform two important roles.

Chemical reactions on the particle surfaces convert stratospheric chlorine from the usual long-lived, inert reservoirs (HCl and ClONO₂) into more active forms. Also, by gravitational settling, the PSC particles remove nitrogen oxides from the lower stratosphere. After air in the polar vortex has been thus primed, with high levels of active chlorine and low levels of nitrogen oxides, ozone destruction can occur when sunlight returns to the region, via catalytic cycles involving ClO (which dimerizes to form Cl₂O₂; ref. 7) and to a lesser extent BrO. Destruction of ozone can continue as long as these elevated levels of ClO and BrO remain.

In contrast, the winter polar vortex in the Northern Hemisphere is warmer and more dynamically disturbed than in the south. Therefore, PSCs are rarer⁸ and the polar vortex breaks down earlier in the season than in the south. For these reasons, ozone depletion seems less likely. However, the flow in the lower stratosphere in the Arctic winter is far less zonal than in the Antarctic, so it is possible for ozone destruction to occur earlier in the season, when the Pole is still in 24-hour darkness, in air parcels transported towards the Equator from cold regions near the Pole to sunlit regions further south.

Although no dramatic loss of ozone has been observed in the Arctic, a gradual long-term downward trend has been reported at northern mid-latitudes⁹. Results from the Airborne Arctic Stratospheric Experiment (AASE) in January and early February 1989 showed that there were high levels of ClO (ref. 10) and

that the vortex was 'primed' for destruction¹¹. Schoeberl *et al.*¹² inferred from the data that some ozone loss (around 20 parts per billion per day or about 1 per cent per day) occurred towards the end of the mission. This was reproduced in trajectory modelling studies¹³. Proffitt *et al.*¹⁴ proposed that the ozone destruction had in fact been much larger but was ameliorated by the poleward flux of ozone-rich air. Much of the destruction, they argued, occurred in the Arctic winter before the AASE campaign started.

Balloon flights

Hofmann and Deshler⁴ conducted a series of balloon flights from 8 January to 8 February 1990 from Kiruna, Sweden (68° N, 21° E). Balloon-borne sensors measured the vertical profile of ozone concentrations up to about 30 km. The authors found a gradual decline in ozone levels during this period, modulated by fluctuations caused by the movement of air over Kiruna. The largest fractional losses, corresponding to about 100 parts per billion per day early in January, occurred at a height of around 22 km. Later this rate fell to 20 parts per billion per day, like that estimated for February 1989.

It is important to determine whether the loss was a manifestation of dynamics carrying ozone-poor air over Kiruna or whether it was due to chemical destruction. Hofmann and Deshler believe it was the latter. They analysed temperature maps of the Arctic region at a height of about 22 km (30 millibars pressure) to determine the movement of air parcels before their appearance over Kiruna. In this way they deduced the amount of time an air parcel had spent below the temperature threshold for PSC formation and how long it had been in sunlight. In general, air parcels arriving at Kiruna had passed close to the cold North Pole, providing the opportunity for chlorine activation to occur. They then emerged into sunlight near Greenland, where catalytic ozone destruction could occur.

Hofmann and Deshler find a strong anticorrelation between the time spent by air parcels below 193 K, at which

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In the works

CONVENTIONAL thinking comes under fire with a report by M. Carmo-Fonseca and colleagues which provides evidence for the existence of RNA-processing 'factories' in the nuclei of eukaryotic cells (*EMBO J.* **10**, 195–206; 1990). It has long been thought that the small nuclear ribonucleoproteins (snRNPs) involved in the splicing of pre-messenger RNA are distributed throughout the nucleus. Not so, say Carmo-Fonseca *et al.*, who using antibodies against snRNP-specific proteins and snRNA antisense probes show that these snRNPs are found mainly at a few distinct sites. The implication is that eukaryotic nuclei contain an organelle that either assembles mature snRNPs or uses them in pre-mRNA splicing.

Something for nothing

If not quite routine, the capture and manipulation of atoms in beams of laser light is nonetheless becoming popular in optics laboratories. But ghostly as these optical forces may seem, they are quite tangible compared with one created from nothing now proposed by S. Haroche *et al.* and by B.-G. Englert *et al.* (*Europhys. Lett.* **14**, 19–24 & 25–31; 1991). The idea exploits the omnipresent electromagnetic fluctuations that characterize the vacuum. In a micromaser, a superconducting cavity selects a single frequency that resonates with the microwave radiation from an excited atom. The ghostly vacuum fluctuation quickly stimulates the atom to radiate. The force now discovered arises if the atomic and cavity frequencies are not quite matched. As the atom tries to radiate into the wrong microwave mode, it is either drawn into the cavity's centre or repelled from it, depending on which frequency is higher.

Bull hit

THE high affinity of the protein, avidin, for its natural ligand, biotin, has given rise to a range of biochemical fancies, the latest of which offers a means of speeding an enzymic bullet to a selected target (E. A. Bayer *et al.* *Biochemistry* **29**, 11274–11279; 1990). Avidin is a tetramer, so when it takes up one molecule of a biotinylated protease, three biotin sites remain vacant. If one of these is allowed to fasten itself onto a biotinylated substrate, proximity ensures rapid attack by the protease, which shows no interest at all in other substrate molecules in the solution. So a working biotinylated enzyme can be brought to a standstill by simply adding avidin. Another way to present the substrate to the enzyme is by way of a biotinylated anti-substrate antibody. The authors envisage applications of their invention to substrates in complex systems — selected constituents of cell surfaces are one example.

temperatures type I PSCs should be present, and the ozone mixing ratio at the same altitude. For these trajectories, however, the amount of sunlight received by the parcels was relatively constant.

This result raises the important question of which factors control polar ozone depletion. The catalytic cycles believed to cause ozone destruction all require sunlight to operate, which is thus a prerequisite. Given this, a key question is the extent to which air can be processed by a cold region containing PSCs and maintain the conditions of perturbed chemistry. In other words, what is the timescale for the deactivation of chlorine after an air parcel has passed through such a cold region? These results suggest that the amount of time air is exposed to PSCs is the most important factor in determining the ozone depletion. The amount of denitrification will depend on this length of time, and

CONSERVATION BIOLOGY

Overgrazing overstated

Ruth Mace

SOMETIMES we are so sure of something that we don't need to see the evidence. That Africa's rangelands are being reduced to desert through overgrazing by domestic livestock is received wisdom. But, as became plain at a recent meeting*, such a view may be seriously flawed.

Conventional thinking is based on two premises. Simple predator-prey models of herbivory illustrate how high grazing pressure can hold a system below its maximum productivity¹. And Hardin's classic paper² on the intrinsic propensity of individuals to overuse resources owned in common explains why herders are likely to push communal rangeland into this overgrazed state. For decades, most pastoral development projects in Africa have been based on the principle that self-interest makes pastoralism underproductive and environmentally damaging. So there has been an emphasis on communal projects, such as group ranches, grazing rotation schemes and reduced herd sizes, on the grounds that short-term inconvenience to herders would act for the greater good. The problem is that these programmes do not work, and generally end in the ignominious retreat of the development agencies.

Words such as 'overgrazing' and 'overstocking' assume that we know what the right stocking density is, which is taken to be when a particular grazing system is at or below its 'carrying capacity'. But great uncertainty centres on the concept of carrying capacity^{3,4}. Population biologists recognize it as the population size at which a population stops growing (*K*). Range

thus so will the rate of chlorine deactivation as ClO is converted to ClONO₂ by reaction with NO₂. Thus, it is not enough for air merely to have encountered PSCs and the total time spent at cold temperatures is important.

Alternatively¹³, the temperature of an air parcel may control the amount of ozone destruction via the thermal decomposition of Cl₂O₂. In warmer conditions, for which this thermal decomposition becomes significant relative to the photolysis of Cl₂O₂, ozone destruction will effectively stop. As Hofmann and Deshler point out, studying the new data with a detailed photochemical trajectory model will help to elucidate the mechanisms controlling ozone loss. □

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scientists, who have generally assumed that environmental damage occurs when livestock numbers exceed carrying capacity, have borrowed the term and given it a different meaning, or rather a range of meanings, which are more akin to an optimal stocking density rather than a maximum (nearer *K*/2 rather than *K* if a population were to grow logistically).

Compounding the confusion is the way range carrying capacity is usually calculated — by estimating the total edible plant biomass produced annually, multiplying it by a 'proper use factor' and dividing it by the amount of forage an animal needs to survive, thereby giving the theoretical number of animals that a range can sustain. All of these measures suffer from variability, error and subjectivity (B. Norton, Utah State University), giving a final figure that it is probably not too cynical to describe as arbitrary. Plant productivity is strongly dependent on rainfall, which varies greatly from one year to the next. The 'proper use factor' is rarely more than a rule of thumb, as the relationship between how much forage is left uneaten and next year's growth is not well understood. Even estimation of the quantity of forage needed by an animal is not straightforward, as it will depend on the economic objectives of the herder. For example, five thin animals may be better than three fat ones if milk and hides are the most important products, which is commonly the case in traditional systems.

Norton illustrated how unreliable this method can be with an example where livestock numbers in Somalia were stated to habitually exceed eightfold the care-

*Savannah Development and Pasture Production, Woburn, UK, 19–21 November 1990.

fully calculated carrying capacity — a biological impossibility. The tragedy of African range management is that so much time and effort has been invested in such calculations, the only consolation being that few attempts to reduce stocking densities to below 'carrying capacity' have ever been carried out, not least because of massive resistance from local pastoralists.

Estimates of carrying capacity are being refined, and there have been many attempts at using methods that do not rely on biomass estimation. But more important is the likelihood that the whole concept of optimal carrying capacity may be inappropriate in huge areas of Africa's rangelands. On the basis of nearly a decade of team work on a Turkana pastoral system in the arid north of Kenya, J. Ellis (Colorado State University) argued that highly variable rainfall rather than livestock numbers is controlling ecosystem dynamics. The conventional view is that livestock overgrazing causes population crashes, and if livestock numbers were below carrying capacity a stable equilibrium could be achieved. But if climate is causing population crashes, the search for a potentially stable stocking density is doomed to fail⁶. The opportunistic strategies of traditional pastoralists, who rebuild herds as fast as possible after drought and are inevitably knocked back in dry years, may be the most efficient use of range. Livestock numbers barely ever reach a level that can seriously harm the range⁶.

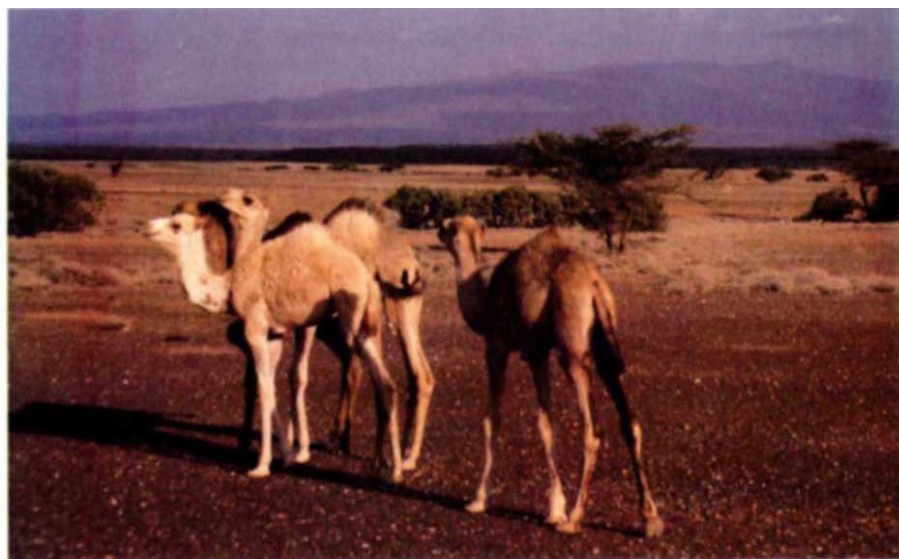
Where changes in vegetation are apparent, it is not clear whether they exist on a significant scale. For example, at the end of the dry season in a grazing system in the Gourma region in Mali, a 15 km grazing orbit around each permanent water source — 40 per cent of the total area — is almost totally denuded (P. de Leeuw, International Livestock Centre for Africa, Bamako). This makes great material for journalists, aid workers and ecologists. But there is so much seed around in this annual grass ecosystem that, at the next rain, all but a small area around the water grows back. The 60 per cent of the range away from permanent water can only be grazed in the weeks before temporary rainwater pools have evaporated and thus cannot be fully grazed. A predominance of annual grass species is sometimes used as a sign of overgrazing. Annual grass is expected in dry Sahelian areas with only one season of rain, however, and should not be considered as an early stage of a classic successional progression from annual to perennial grass and forest, held back by livestock overgrazing (N. de Ridder, University of Groningen).

Although these arid systems may be more resilient than we had thought, range can undoubtedly be pushed to a point from which recovery is hard. Heavy

trampling by livestock on tracks and by water can prevent plant growth and destroy soil structure. Westoby *et al.*⁸ believe that an appropriate model of succession in African rangeland is one in which some states may be stable in face of considerable pressure. But once pushed beyond a certain point, the range may undergo a relatively rapid transition to another state which also has its own local stability. Climatic events or human action (such as fire) may be agents of transition. The management implications of this view are that action would be useful only at points of particular pressure. Maintaining and

centuries of environmental flux. Billé has suggested that, as the bush matures over years, nutrients are returned to the soil through leaf litter, stands eventually open up as tree canopies develop, and perennial grass and fire return. The cycle may take 60 to 100 years.

Proponents of the chronic overgrazing story argue that pastoralists are progressively destroying their resource base^{9,10}. If rangeland degradation is occurring on a large scale, then livestock numbers should be in longterm decline. There is no evidence for this — indeed the converse is true in many areas. Ironically, those eco-



Overgrazed or naturally arid? Rangeland in northern Kenya in the dry season.

encouraging herd mobility may be important in this respect, but general destocking would probably be pointless as livestock are generally too widely dispersed over most of the range to cause a transition from one state to another.

In perennial grasslands, the story may be different. Heavy grazing does tend to have a more dramatic effect on plant communities in these wetter semi-arid and sub-humid savannahs, and classic models of continuous growth and succession may have more relevance. L. Coppock (International Livestock Centre for Africa, Addis Ababa) reported on another ten-year study on the Borana plateau in southern Ethiopia, which is wetter than the Turkana ecosystem and has been inhabited by pastoralists for centuries. J. Billé has found that in areas of heavy grazing, perennial grasses lose out against woody seedlings, the risk of fire is reduced and bush encroachment follows. This eventually is a cattle-rancher's nightmare, although good news for browsers such as camels and goats, which are frequently important components of traditional pastoral systems. Multi-species herding is the norm in Africa; indeed, switching emphasis between species in the herd may be one of the ways pastoralists have adapted to

systems where drought and high livestock mortality are most frequent may be those that are the most resilient. It would be irresponsible to claim that no long-term damage is being done, and that was not the conclusion of the meeting. Rather, the message is that our knowledge of range ecology is too incomplete to justify deflecting development aid away from supporting a system that may be more efficient and less damaging than irrigation and agriculture. □

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Wondrous transformation

Michael Akam

VERTEBRATES and insects have similar clusters of homeobox genes, the protein products of which play a crucial part in determining the course of development¹; in insects at least, these proteins serve as labels that define a cell's position along the body axis^{2,3}. Striking parallels between the vertebrate *Hox* and insect homeotic gene clusters suggest that they have a common origin, and may possibly retain vestiges of common function (see figure). That possibility has now been tested and confirmed in a series of audacious gene-swap experiments reported in *Cell* by McGinnis and his colleagues^{4,5}. They have expressed in *Drosophila* the proteins encoded by two vertebrate *Hox* genes, and find that each of these proteins induces a specific developmental response characteristic of the product of the related gene in *Drosophila*.

The perfect way to test the functional equivalence of gene products in two different species is to place the coding sequence from one into the normal chromosomal environment of the other, under the regulation of an endogenous promoter. This is not yet possible for the homeotic genes. McGinnis and his colleagues have therefore placed the vertebrate *Hox* genes under the control of a heat-inducible promoter, and used a transposable P element to insert this construct into the fly genome.

Drosophila homeotic genes have previously been placed on similar constructs. When strains carrying such constructs are subjected to heat shock, the homeotic protein is transiently expressed at high levels in all cells of the developing embryo⁶⁻⁸. This does not mimic normal expression, but it does result in a suite of developmental changes that is specific for each protein and that is clearly related to the normal developmental effects of that protein. For example, the *Antennapedia* (*Antp*) gene is normally expressed in the thoracic segments, where it promotes the

development of, amongst other things, legs. In a heat-shock-promoter/*Antp* construct, as in certain other *Antp* mis-expression mutants, the normal *Drosophila* protein is expressed in the head, causing the antennae to develop as legs⁹. When McGinnis uses the mouse *Hox-2.2* construct instead of the *Antp* gene, he sees the same transformation.

Antennapedia is not the only *Drosophila* gene that can induce antenna-to-leg transformations. Leg development can also be induced by expressing the Ultrabithorax homeoprotein in developing antennae¹⁰, but the leg structure, and other transformations induced in the developing embryo, are different from those induced by the *Antp* product. *Hox-2.2* mimics at least some of these specific effects of *Antp*. An equally specific effect is seen when the human counterpart of the *Deformed* (*Dfd*) gene is expressed in flies — in this case an effect on head morphology, which closely mimics the original *Dfd* mutant⁵.

There are two ways in which these effects may be brought about. The most obvious is that the induced vertebrate protein may interact with the normal downstream targets of the equivalent protein in *Drosophila*. However, an alternative mechanism is suggested by earlier work with the *Drosophila* heat-shock constructs⁷. The induced homeoprotein may activate the resident copy of the corresponding *Drosophila* gene, stimulating an autoregulatory pathway. This appears to be how a heat-induced pulse of the *Drosophila* *Deformed* protein affects development. In certain cell types, a brief pulse of *Deformed* protein is sufficient to turn the normal *Dfd* gene on inappropriately. It is in these cells that a homeotic transformation occurs.

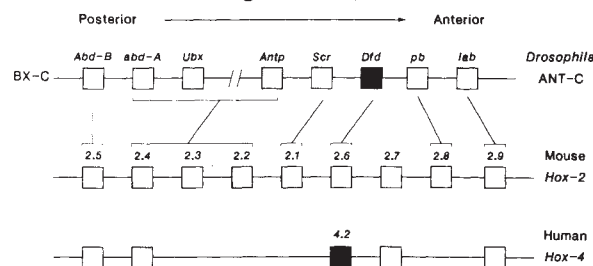
The human counterpart to the *Deformed* protein, the product of the *Hox-4.2* gene, appears to work in the same way. It activates the *Dfd* gene in *Drosophila*, but it does not activate any of several other homeotic genes tested⁵ (thereby further demonstrating the specificity of the heterologous interaction). *Antennapedia*^{6,8} and its protein counterpart in the mouse do not seem to act in this way, for the effects of mis-expression are seen even

in embryos that lack a normal copy of the *Antp* gene. Presumably in this case the vertebrate gene is able to interact with at least some of the normal downstream target genes. Why the responses to *Deformed* and *Antennapedia* protein should differ in this way is not clear but, in each case, the regulatory effects of the vertebrate products mimic those of their counterparts in *Drosophila*.

The clearest conclusion to be drawn from these experiments is that the structural similarities between the homeobox genes in vertebrates and those in insects do translate into similar regulatory specificities. This was not a foregone conclusion, for besides the homeobox and its immediately flanking sequences, only short peptide motifs are generally conserved between the proteins in *Drosophila* and vertebrates¹¹.

A distinct question is whether the similar vertebrate and insect proteins are functioning within a network of conserved regulatory interactions, in which both upstream regulators and downstream targets are the same in different species. The new results do not prove that, but as the extent of functional similarity between individual proteins is revealed, so the likelihood of such conservation is increased. It may be humbling to discover just how few changes nature has needed to mould man and fly from their common ancestor. □

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Drosophila genes and vertebrate *Hox* genes tested for function in the gene-swap experiments of McGinnis and colleagues^{4,5}. The homeotic genes of *Drosophila* occur in two separate clusters on the third chromosome (ANT-C and BX-C), but probably derive from the splitting of a single ancestral cluster¹¹. The chromosomal organization of the vertebrate *Hox* genes resembles that inferred for the ancestral insect cluster, but this ancestral unit appears to have been replicated in the lineage leading to mammals, so that each genome contains four clusters which have a similar overall structure¹²⁻¹⁴. (Specific genes appear to be missing or duplicated in individual clusters.) Two of the four clusters are shown here (modified from ref. 12). Genes at corresponding positions in each vertebrate cluster show extensive sequence similarities. These subsets are termed paralogous loci (examples are *Hox-2.6* and *Hox-4.2*). Comparisons of the sequences of the vertebrate and *Drosophila* gene products allow specific sets of paralogous vertebrate genes to be matched with a particular gene in *Drosophila*¹⁰ (for example the *Hox-2.6* family with *Deformed*). In the diagram, lines connect the *Drosophila* genes with their counterparts in the vertebrate complexes. Such matching reveals that the linear order of the genes within clusters has been conserved between vertebrates and *Drosophila*. In both vertebrates and *Drosophila*, the genes of each cluster are activated in a series along the anteroposterior axis of the body. This series parallels the order of the genes on the chromosome, so a particular vertebrate gene resembles its equivalent gene in *Drosophila* not only in sequence, but also in being activated at a similar relative position along the body axis¹²⁻¹⁴. Shading marks the genes used in the gene-swap experiments described here.

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Deep mantle melting

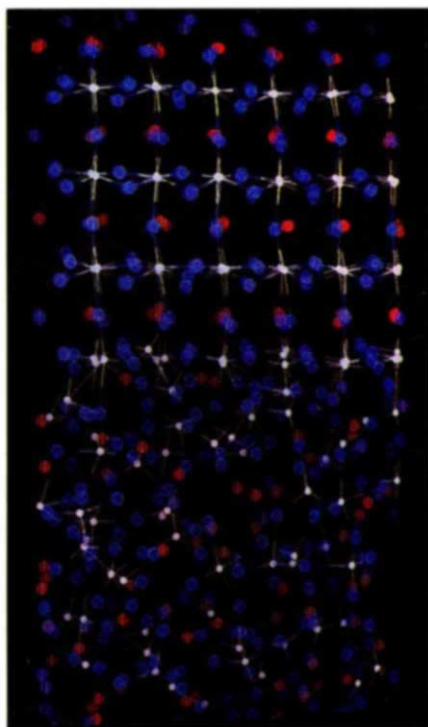
R. J. Hemley and J. D. Kubicki

WHEN melting of common silicate minerals occurs at or near the Earth's surface, the density of the melt is less than that of the solid phases from which it was derived. But at very high pressures, for example at the pressures of the Earth's mantle and core, what happens can be very different. Under these conditions the density of the melt approaches, and may even exceed, that of the corresponding crystalline phases with which it is in equilibrium¹. A condition of equal buoyancy could be reached: melts below a specific depth would sink; above it they would rise. This would have significant implications for the manner in which the Earth has evolved because it would impede heat transfer from the interior and alter the style of geochemical differentiation of the planet as a whole. Although there is growing experimental evidence of such behaviour, direct experimental studies of deep mantle material at the appropriate pressures and temperatures have not been entirely definitive, and theoretical approaches have not been sufficiently accurate for quantitative predictions. A new theoretical study by Stixrude and Bukowinski² represents an important step towards elucidating the behaviour of silicate melts in the Earth's deep interior by providing a thermodynamic framework in which the compression of these liquids may be understood.

Stixrude and Bukowinski apply a simple thermodynamic formulation to the problem of predicting what will happen at pressures beyond the range for which experimental data are available — such as at the base of the lower mantle, where pressures reach 136 gigapascals (GPa). The approach is purely phenomenological: the Helmholtz free energy, which is a function of the volume and temperature, is parameterized to fit available experimental high-pressure data (principally phase equilibria and equations of state). The approach is tested by fitting the melting relations for simpler materials, such as liquid alkali metals and water, which are relatively well characterized. The authors then go on to silicates, fitting the low-pressure phase relations and thermodynamic properties such as the thermal expansivity and heat capacity. The known melting relations of silicates at upper mantle pressures are reproduced accurately and agree with similar phase-equilibria calculations carried out for this range by Fei *et al.*³.

But Stixrude and Bukowinski's primary concern is melting of silicate material at much higher pressures, as pertain throughout the lower mantle, which extends from 650 to 2,900 km depth,

including the D'' region, a 200-km-thick, seismically distinct layer that surrounds the iron-rich molten outer core. High-pressure experiments, in combination with seismic density profiles, indicate that (Mg,Fe)SiO₃ in the perovskite structure is the dominant mineral of much of the



Molecular-dynamics simulation of MgSiO₃ at high pressures and temperatures showing melting at the perovskite–liquid interface: magnesium (red), silicon (white), oxygen (blue). The coordination of the silicon is predominantly tetrahedral in the liquid but octahedral in the crystalline phase. (Density $\rho \approx 4.25 \text{ g cm}^{-3}$, pressure $P \approx 40 \text{ GPa}$, temperature $T = 2,500 \text{ K}$.)

lower mantle. Stixrude and Bukowinski predict that the melting temperature of MgSiO₃ perovskite is essentially independent of pressure above about 50 GPa and has a weak maximum; it is also at least 500 K above the expected temperatures of the lower mantle, which is consistent with seismological studies that show no evidence for widespread melting.

The results are largely in accord with the pioneering diamond-cell studies of perovskite melting by Jeanloz and colleagues^{4,5}. The calculated density changes for the liquid are consistent with those determined⁶ for molten diopside (CaMgSi₂O₆) to 38 GPa using shock-wave techniques. There is thus compelling evidence from static high-pressure experiments, shock-wave studies and thermodynamic modelling that the compression of such basic melts in the mantle

is appreciable: even if there is no crystal–melt density inversion, the density contrast between solid and liquid may be negligible throughout much of the lower mantle.

The detailed mechanism by which the melt can achieve such high densities cannot be directly addressed by an appeal to thermodynamics. It has long been thought that changes in cation coordination number that accompany pressure-induced polymorphic transitions in the crystalline minerals are associated with similar coordination changes, albeit continuous, in high-temperature melts. Our recent spectroscopic studies⁷ of the metasilicate glasses at mantle pressures, however, indicate that tetrahedral silicon can be preserved to relatively high pressures (above 35 GPa), with the compression achieved mostly by repacking and distortion of SiO₄ tetrahedra. Recent molecular dynamics simulations of the melts show that, over an appreciable range of mantle pressures, silicon in liquid MgSiO₃ is predominantly in tetrahedral coordination (see figure), with increases in coordination occurring gradually over a wide range of pressure (see ref. 7). Evidently, the higher degrees of the freedom available in the liquid and glass permit higher densities relative to the crystal without necessarily increasing silicon coordination.

So far, all such studies of melting in the deep mantle are subject to large uncertainties; and the recent thermodynamic calculations are no exception: there is still the problem inherent in extrapolating empirical data beyond the measured range. The melting interval for perovskite at the pressures of D'' is calculated to be below the maximum temperatures in this layer, estimated⁵ to be 4,500–5,000 K at the core–mantle boundary (see refs 2 and 5). Stixrude and Bukowinski thus argue that perovskite is unstable in the D'' layer and (since the layer is largely solid) the material dissociates to form simple oxides (Mg,Fe)O and SiO₂. The calculated melting curve of the silicate perovskite at these pressures, however, is strongly dependent on the form of the extrapolated pressure–volume–temperature equations of state for both solid and liquid.

Notably, there have been no direct equation-of-state measurements for MgSiO₃ liquid at high pressure. Accurate measurements for the silicate perovskite

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have been carried out, but there is a need to extrapolate the effects of thermal expansion to lower-mantle temperatures (above 1,700 K). Small differences in thermal expansivity affect the densities, and hence the shape of the melting curve. Our new equation-of-state measurements⁸ indicate that the thermal expansion at high pressure is smaller than that predicted by the model⁷; this results in a positive slope of the calculated melting curve for the conditions in the lower mantle and hence a higher likelihood of stability of MgSiO₃ perovskite in the D'' layer. This result appears to be in better agreement with the most recent melting data⁵ for (Mg,Fe)SiO₃.

There is thus a need for more measure-

ments and theoretical studies of equations of state and melting relations under deep mantle conditions. The effect of other components (particularly Fe, but also Ca, Al and H), as well as the very important possibility of incongruent melting, also needs to be determined. In a broad sense, the noteworthy accomplishment of the recent thermodynamic studies^{2,3} lies in pointing out that a quantitative description of silicate phase relations throughout the ranges of pressure and temperature of the upper and lower mantle may be within reach. □

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Inflammatory activities

Peter J. Barnes

LIPID mediators, such as prostaglandins, leukotrienes and platelet-activating factor (PAF), which are generated from membrane phospholipids by the action of phospholipase A₂, have potent inflammatory effects and are likely to have an important role in inflammatory diseases. All of these lipid mediators produce their effects by activating specific cell-surface receptors, which have been extensively characterized by pharmacological techniques. But, until now, no one had cloned a lipid-mediator receptor. On page 342 of this issue¹ Honda *et al.* report the cloning and expression of a complementary DNA for a PAF receptor from guinea-pig lung. They used a ligand-specific cloning strategy which involves expression of the cDNA in *Xenopus* oocytes, and reveal that the receptor is yet another member of the superfamily of receptors coupled to GTP-binding proteins (G proteins).

Platelet-activating factor is a potent mediator of inflammation, causing microvascular leakage, vasodilation, contraction of certain smooth muscles, endothelial adhesion and activation of several types of inflammatory cells, such as macrophages, neutrophils and eosinophils². It is synthesized and released from several types of inflammatory cell and also activates the same cells. Because it is rapidly metabolized, it may function as a locally acting or 'paracrine' mediator. Because of its many effects, PAF has been associated with a host of human diseases³. It is implicated in the pathophysiology of asthma, particularly in the pathogenesis of the chronic eosinophilic inflammatory reaction in the airway mucosa, which may underlie airway hyper-responsiveness, a key abnormality of asthma⁴. Indeed PAF more closely mimics the pathological features of asthma than any other single mediator. Studies in unaffected human

volunteers and in several other species have demonstrated that, when inhaled, PAF can increase airway responsiveness for prolonged periods⁴. There is also good circumstantial evidence that the factor mediates some of the features of septicaemic shock⁵.

The actions of PAF are mediated through specific surface receptors, although there is some evidence that accumulation of intracellular PAF (which represents the majority of PAF synthesized in a cell after activation in many cell types) may also influence cell function⁶. PAF receptors have proved difficult to study in several types of cell or tissue when [³H]PAF has been used as a ligand, with often conflicting results from different laboratories⁷⁻⁹. This may be because [³H]PAF is taken up by cells and rapidly metabolized; is lipid soluble, giving rise to high nonspecific binding; and may itself induce downregulation of receptors. These problems have been overcome by the introduction of labelled antagonists, the most useful of which seems to be [³H]WEB 2086, which has a relatively low nonspecific binding related to its low lipophilicity. [³H]WEB 2086 has been used to demonstrate PAF-binding sites, with the characteristics expected for a receptor, in several inflammatory cells¹⁰⁻¹², and in human and guinea-pig lung^{13,14}. But because of a lack of suitable photoaffinity ligands, it has not so far been possible to purify and isolate the PAF receptor, the first step in obtaining the sequence.

The intracellular pathways involved in PAF signalling involve activation of a G protein. This has been demonstrated by the inhibition of PAF binding by GTP^{15,16} and by its stimulation of GTPase activity in membranes^{15,17}. The hydrophobic profile of the PAF receptor, with seven putative membrane-spanning α helices, is typical

of other G protein-linked receptors. But the nature of the G protein is far from certain. This might be because PAF receptors are linked to different intracellular pathways. In rabbit neutrophils pertussis toxin inhibits both phosphoinositide hydrolysis and degranulation caused by PAF, indicating that the G_i type of G protein is involved, whereas mobilization of calcium ions is unaffected¹⁸. In human neutrophils, PAF-stimulated GTPase activity is inhibited by both pertussis toxin and cholera toxin, indicating that both G_i and G_q proteins are present, whereas in human platelets neither toxin has an effect¹⁵. The cloned PAF-receptor cDNA reported by Honda *et al.* does not give any clue as to which G proteins are coupled, but the functional studies indicate that subtypes of the receptor may exist.

The whole question of PAF-receptor subtypes is controversial, as there are no clearly selective drugs for them. Several molecular species of PAF have been isolated, with the C₁₀ species predominating¹⁹, but there is no convincing evidence that they act on different receptors. Differences in the potency of PAF on different cellular functions and the differential sensitivity of the PAF response to cations provide weak evidence that subtypes of the receptor exist²⁰⁻²². The cloning of a PAF-receptor cDNA may now lead to the discovery of unsuspected subtypes, as has been the case for many other receptors,

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such as muscarinic receptors. The receptor cDNA hybridized with messenger RNA in leukocytes, lung, spleen and kidney, indicating that the same receptor is likely to be present in these organs. The cellular localization of receptor transcription is not yet known however.

The amino-acid sequence of the PAF receptor, deduced from the sequence of its cDNA, has some interesting properties. The aspartic-acid residue of the second transmembrane helix, the cysteine residues on the second and third extracellular loops, and the three proline residues in the sixth and the seventh transmembrane helices, may contribute to the ligand-binding pocket, which has been described for β -adrenoceptors²³. The four serines and five threonines on the cytoplasmic tail may be sites of phosphorylation, possibly involved in the progressively smaller response of many cells to repeated exposure to PAF, a phenomenon that apparently occurs in oocytes injected with PAF-receptor mRNA.

The cloning of a PAF-receptor cDNA

opens up new avenues of research. Factors which regulate gene expression of the receptor may now be investigated, and the distribution of the receptor studied by *in situ* hybridization. The availability of the cloned receptor cDNA may also lead to a better understanding of the coupling of PAF receptors. Although it is commonly suggested that cloning of the cDNA for a receptor may help in the design of new therapeutic agents, this is unlikely to be the case, because the corresponding amino-acid sequence does not provide sufficient information to work on. But knowledge of the sequence of the gene encoding the PAF receptor may allow modified oligonucleotides to be developed that suppress receptor expression. Undoubtedly, reports of the cloning of the cDNAs for other lipid-mediator receptors will follow. This will certainly lead to a better understanding of inflammatory diseases and their control. □

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BIOMINERALS

Flattery by imitation

Stephen Mann

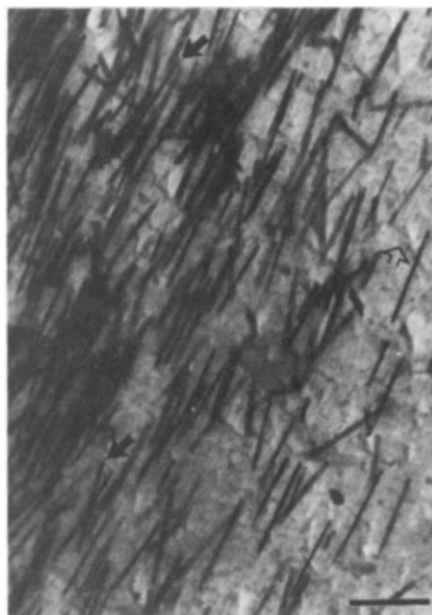
THERE is a growing awareness in materials science that the adaptation of biological processes may lead to significant advances in the controlled fabrication of superior composites, ceramics and polymers¹. One possible approach to so-called smart materials now receiving serious attention is the synthetic mimicking of biominerals such as bone, teeth and shells. On page 315 of this issue², Bianconi and colleagues describe such mimicry in the synthesis of a composite material in which cadmium sulphide crystals are embedded in a polymeric matrix.

The properties of the natural biominerals are attributable to their complex, highly organized composite structures, in which inorganic crystals are dispersed within organic polymeric matrices. In bone and related tissues, plate-like calcium phosphate (carbonated apatite) crystals are aligned within a collagen matrix so that the mechanical properties of the material are dependent not only on the matrix-mineral composition but on the type and level of organization. Mature tooth enamel also consists of a calcium phosphate/organic matrix composite but the inorganic crystals are long thin needles and the matrix is an assembly of acidic macromolecules, termed enamelines.

Shells, by contrast, are organized composites of calcium carbonate (calcite and aragonite) crystals interspaced within a complex matrix of glycoproteins rich in acidic amino acid residues. Depending on

the arrangement of crystals, nacreous, prismatic, foliated and crossed-lamellar layers are constructed and these different types of ultrastructure are related to changes in the composition of the associated organic matrix³.

A key feature of biological composites



A thin section of a limpet tooth showing needle-like crystals of the iron oxide, goethite (α -FeOOH). The crystals are aligned within a polysaccharide organic matrix (open arrow). Scale bar, 0.6 μ m.

is that they have a remarkable level of control with regard to the size, shape and organization of crystals within the organic matrix (see figure). How this is achieved remains unclear although it is generally accepted that the matrix plays a role in the *in situ* nucleation and growth of the mineral phase. One possibility is that the chemical structure of the matrix surface complements a specific crystal face of the mineral so that nucleation is directed along a preferred crystallographic axis. This implies that the matrix macromolecules contain domains of charged residues arranged periodically at the mineral-matrix interface. Mimicking this process within a synthetic composite is not trivial, as controlling the nature of interfaces within polymeric matrices is notoriously difficult. Thus, the ability to produce man-made analogues of biominerals would represent a fundamental breakthrough in materials synthesis. The work carried out by Bianconi and colleagues² is notable in going a significant way towards achieving this aim.

There have been a number of reports of composites made by the *in situ* reaction of metal salts and complexes in polymers. These include iron oxides in poly(methylmethacrylate)⁴ and polyvinylidene fluoride⁵, silica in silicone rubbers⁶, cobalt metal in various polymers⁷, copper and cadmium sulphides in polyacrylonitrile⁸ and copper metal in cellulose⁹. In these systems, the polymeric host often constrains the size of the inorganic particles and this is often a viable route to the preparation of 'nanophase' materials. However, there is little evidence of any effect on the crystal shape and orientation of the guest particles.

The new work of Bianconi *et al.*, in which CdS particles are formed within poly(ethyleneoxide) (PEO) films, shows how composites with controlled properties can be prepared by the judicious choice of synthetic conditions. The approach aims to mimic three fundamental aspects of biomineralization: preorganization of the matrix; strong molecular interactions between the matrix and inorganic reactants; and a controlled flux of reactants at the nucleation site. Using PEO is of key importance as it readily forms semicrystalline films in the presence of aqueous metal salts such as CdCl₂ — a method analogous to the possible ordering of β -pleated sheet motifs within biological matrices by Ca²⁺ ions before calcification. The next step is the room-temperature reaction of the partially organized Cd-bound film with a solution of S(SiMe₃)₂ in tetrahydrofuran. A slow reaction rate (7 days) allows the organosulphide to penetrate deep into the polymeric matrix. This prevents the growth of an impermeable layer of the reaction product at the film surface, so that CdS particles are dispersed homogeneously

throughout the polymer. Furthermore, by controlling the CdCl_2 concentration, particle sizes can be varied from 2 nm to 2 μm .

So far, so good. The synthetic system of Bianconi *et al.* successfully amalgamates several aspects of biomineralization and shows how these factors induce regularity in the site of CdS deposition. But the real test of how good the system is rests with the level of control of crystal shape and orientation. Here, the initial results were disappointing. X-ray and electron-diffraction studies indicated that the CdS particles are noncrystalline, suggesting that the PEO matrix acts as a potent inhibitor of CdS crystallization at room temperature.

Heating the composite film to 120 °C for 1 hour, however, changes the amorphous material into regular cubic crystals dispersed within the polymer matrix. Interestingly, this phase transformation is influenced by the crystallinity of the polymer, as the process of direct reaction at 120 °C of an amorphous PEO/ CdCl_2 film with $\text{S}(\text{SiMe}_3)_2$ gave crystalline CdS of random morphology. The authors suggest that ordered aggregation of the amorphous particles induced by the breakdown of the semicrystalline matrix at 120 °C is responsible for the regular crystal size and habit of the final product. This is an interesting speculation and one that may have biological relevance since many organized, crystalline biominerals are formed through the phase transition of amorphous precursors. Could conformation changes in biopolymer matrices induced, for example, by post-depositional crosslinking or degradation, activate these processes?

Clearly, the formation of amorphous CdS rather than an oriented crystalline phase in PEO at room temperature necessitates a re-think of the biomineralization analogy. One critical feature to be added is the generally accepted idea in biomineralization of a multicomponent organic matrix comprising structural and functional units. In the experiments described above, PEO can be considered as a structural matrix in the sense of delineating the environment for CdS nucleation and growth. Additional components that interact directly with the formation of nuclei and their subsequent growth are required. Bianconi *et al.* achieved this by repeating the room temperature experiments in the presence of an $\text{S}(\text{SiMe}_3)_2$ solution containing the surfactant aerosol-OT which has a charged sulphonate headgroup. The effect is dramatic. First, crystalline CdS forms within the film. Second, the crystals have a regular cubic morphology. Third, they have the rock-salt structure commonly considered to be the high-pressure phase of CdS. And finally, and most remarkably, the single crystals are oriented with

their a -axis perpendicular to the plane of the organic film.

What is the role of aerosol-OT in these experiments? The authors offer a tentative explanation based on recent studies of controlled crystallization under compressed Langmuir monolayers. These investigations have shown that organized assemblies of surfactant molecules can induce the oriented nucleation of amino acids¹⁰, calcium carbonate¹¹ and ice¹² at air/water interfaces. This specificity arises from geometric, stereochemical and electrostatic relationships between the charged or polar headgroups of the surfactant film and molecules or ions in the crystal surfaces of nuclei forming at the air/water interface. Similar effects may be responsible for the oriented nucleation of CdS in the new system: aerosol-OT molecules could be aligned within the semicrystalline PEO matrix by polar interactions and thereby act as organized nucleation centres for the {100} face of CdS.

This new work offers exciting prospects for materials and polymer scientists. Clearly, many other inorganic materials and crystalline polymers need to be investigated before any realistic appraisal of its technological potential can be considered. One challenge will be to increase the low volume fractions of inorganic particles generated by current batch methods of *in situ* crystallization. Biological systems have solved this problem through the evolution of flow processes whereby cells regulate the fluxes of ions and molecules to and from mineralization sites. Moreover, the physical and chemical status of the biomineral may itself be an important factor in optimizing its subsequent growth and form in time and space. Analogous concepts of dynamics, feedback and iteration in materials synthesis are figments of our imagination. As far as the natural world goes, there can be no doubt that even our perceived 'smart' materials remain firmly entrenched at the bottom of the class. □

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Fixed air

SYNTHETIC polymers have hosts of important uses; but they are a great nuisance once that use is over. Some can be depolymerized back to raw monomer for re-use, though this is seldom economic. Others are designed to rot or biodegrade naturally; so far these are little used. The ideal disposable polymer would depolymerize naturally and spontaneously back to a harmless gas. One candidate for this difficult role, says Daedalus, is polymeric carbon dioxide.

Many polymers, like polyesters, polycarbonates and polyanhydrides, incorporate $-\text{CO}-\text{O}-$ links in their backbone. Polymeric carbon dioxide, the ultimate polyester, would consist entirely of such links. But how to make it? Most polymers are made by highly subtle catalysts, often discovered only by chance. This chance, says Daedalus, may already have been seized by the vegetable kingdom.

He points out that the photosynthesis of atmospheric carbon dioxide, the crucial reaction in all green plants, ceases at night-fall for lack of light. Any plant that continued to absorb carbon dioxide during the night, ready for photosynthesis next day, would have a powerful advantage. Almost the only way to achieve this would be reversible polymerization of the carbon dioxide. So DREADCO botanists are studying the plants of the tropics, where light is plentiful but carbon dioxide scarce, in search of CO_2 -polymerizers. This botanical quest may reveal the crucial enzyme, carbon dioxide polymerase, around which an industrial catalyst can be designed. It should also yield samples of the polymer itself, for study and characterization.

"Vanishing Plastic" (as the product will be called) will need careful formulation. In water or moist air it will slowly hydrolyse, evaporating back to carbon dioxide gas. Its life could be prolonged as desired by terminating its polymer chains, and punctuating them at intervals, with suitably unreactive copolymer groups: a strategy used to stabilize the closely related acetal resins. Short-lived Vanishing Plastics will take over the wrapping and packaging markets; longer-lived ones will be formed into boxes, bottles, pens and so on. Utterly harmless and nonflammable, Vanishing Plastic will be the ultimate environmentally virtuous product. Litter-louts will toss it gaily out of car windows while farmers spread it carelessly on their fields as a combined mulch-film and carbon dioxide source for their crops. Vanishing Cloth will be ideal for nappies, tissues and cleaning-wipes, while Vanishing Paper will form the world's bus tickets, posters, junk mail, office bumph, newspapers and so on — not to mention frank confessions, forged cheques and declarations of undying love.

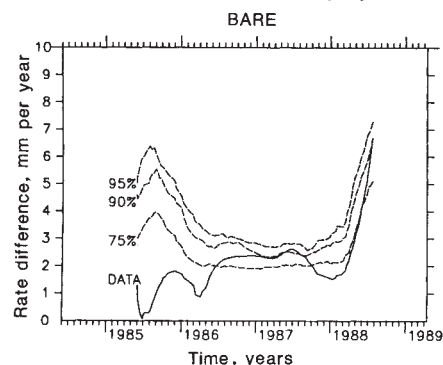
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Earthquake explanations

SIR—In their analysis of the deformation rate across the San Andreas fault near Parkfield, California, Wyss *et al.*¹ did not consider some factors contributing to the uncertainty of the geodetic data and that diminish the significance of the purported anomalous change in deformation rate. For the two geodetic baselines that they studied, I have determined that the rate change is only marginally significant at the 80% confidence level when several factors are considered.

The most significant rate change I found using the procedure of Wyss *et al.* occurred in early 1987 rather than mid-1986. I used 200 synthetic time-series with statistical noise properties similar to those of the actual observations. For the two geodetic baselines, BARE and CAN, I simulated the time-series by partitioning the variance of the observations as the sum of three known noise sources: periodic seasonal signal; spectral white noise, representing instrument precision; and spectral f^{-2} noise, representing a combination of local monument noise and episodic tectonic displacements. Each noise source contributes roughly 0.9 mm scatter in the observations, while the long-period (f^{-2}) noise affects variations in rate². Using the procedure of Wyss *et al.* to fit two linear trends to my synthetic



data, I obtained confidence levels for the difference between secular rates estimated over the intervals before and after each specified date (see figure). Thus, for the baseline to BARE, the highest confidence level occurs during the first half of 1987 and just exceeds the rate difference obtained from 90% of the synthetic data. However, for the rate change noted by Wyss *et al.* for August 1986, the confidence level is only 80%. For the baseline to CAN, the highest confidence level of 90% occurred in January 1987; the August 1986 anomaly cited by Wyss *et al.* exceeds only the 75% level.

I analysed a subset of the data used by Wyss *et al.* because the distance-measuring instrument they used to make the measurements had a series of problems after mid-April 1989 (ref. 3). The measurements made during the summer

of 1989 showed that all baselines contracted by 2.5 mm, but those made during the winter of 1989–90 showed more scatter than normal (see Figs 1 and 3 of ref. 1). When I included measurements after mid-April 1989, the confidence level for the rate change at BARE fell to less than 75%.

Finally, Wyss *et al.* failed to discuss several other factors that further decrease the significance of the rate change. A possible man-made offset in distance data to CAN in late 1985 (ref. 4) contributes a substantial uncertainty to the estimate of the secular extension rate. The average annual rainfall decreased by 30% for the period spanning the data⁴, rather than the 10% claimed by Wyss *et al.* It is possible that the decrease in apparent tectonic deformation rate could be an artefact of

decreasing soil moisture capable of displacing geodetic monuments.

Further, earthquakes outside the Parkfield preparation zone can affect the deformation rate at Parkfield. An example is the Kettleman Hills earthquake of 1985, which produced significant inflections on a few geodetic baselines at Parkfield. Before stating that the deformation at Parkfield changed in mid-1986, Wyss *et al.* should have examined the data for alternative mechanisms and noise sources.

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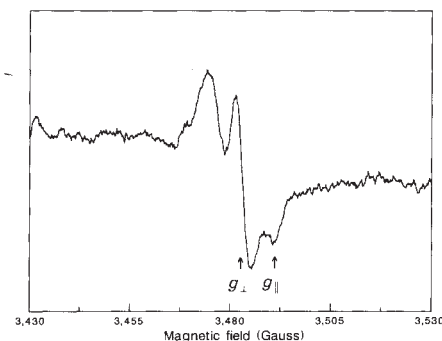
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Radiation doses

SIR—Bone-seeking radiopharmaceuticals are receiving considerable attention for therapeutic and palliative applications, including synovectomy (relief of arthritic pain) and palliative treatment of bone metastases for people with terminal cancer. Candidate radionuclides include phosphonate complexes of samarium-153, rhenium-186 and holmium-166. Clinical trials for human subjects are under way with the first two of these (ref. 1 and R. A. Holmes, personal communication).

The dosimetry for these agents relies on an accurate measurement of the activity administered, and calculations of the dose to the target organ (bone) as well as other organs, following the schema developed by the Medical Internal Radiation Dose (MIRD) committee². The critical data in this model are the physiological uptake parameters for the bone and other key organs, such as the liver and kidneys. (Such data are often available only from biodistribution studies in animals.) If the desired result is not ablation of the bone marrow, then the dose is of critical importance in limiting suppression of the immune system. The National Council on Radiation Protection and Measurements has recommended that experimental methods be developed to validate dose calculations from the MIRD model³.

We report here a new approach to experimental assessment of the radiation dose to bone using electron paramagnetic resonance (EPR) spectrometry. Ionizing radiation generates short-lived ($< 1 \mu\text{s}$) free radicals in soft tissues. In contrast to this, ionizing radiations which interact with mineralized bone tissue (hydroxyapatite) result in the production of a dose-dependent concentration of long-lived (months to years) paramagnetic centres. The hydroxyapatite centre,



We injected a beagle with 20 mCi kg^{-1} holmium-166 ($t_{1/2} = 26.8 \text{ h}$) as the ethylenediaminetetramethylenephosphonate complex. The animal was killed 16 days after the injection, and a humerus removed for study. Conventional dose calculations indicated a 30-Gy dose to the compact bone. We cut a fragment from the whole bone (approximate dimensions $3 \times 25 \text{ mm}$) and put it in a 5-mm quartz tube in an X-band microwave resonator of an EPR spectrometer. The resultant field-swept first derivative EPR spectrum is shown. The characteristic resonances due to the hydroxyapatite paramagnetic centre are designated as $g_{\perp}$ and $g_{\parallel}$ (g is the spectroscopic splitting factor). The ESR signal intensity of these resonances is a well-behaved function of absorbed dose in the bone tissue. The resonance downfield from and partially obscured by $g_{\perp}$ is assigned to an organic radical, and is not considered useful for dosimetry.

detected by EPR, has been used as a marker of radiation exposure and a measure of the absorbed dose⁴. We have applied the EPR technique to bone tissues of an animal treated with a radiopharmaceutical to demonstrate its sensitivity towards radiation-induced centres in the mineralized tissue (see figure). We are now attempting to quantify the EPR spectral intensity in terms of absorbed dose.

Although the EPR bone dosimetry method is invasive, it does offer the first experimental technique for measuring

and mapping the tissue response to the administered radiation. Given the extreme sensitivity of the bone marrow to ionizing radiation, the promise of this technique for improving bone dosimetry (for beam therapy, as well as for bone-seeking radiopharmaceuticals) should be explored.

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Oldest bone disease

SIR—We have identified calcium pyrophosphate deposition disease (CPPD) in Neanderthal skeletons from two widely separated sites. We found classic radio-carpal and tibiotarsal articular surface reflections on the bones of specimens from la Chapelle-aux-Saints (France) and Shanidar (Iraq), which to our knowledge is the earliest documentation of this disorder in humans. As CPPD may be a familial disease, populations from the two sites may be more closely related than has previously been suspected.

We assessed skeletal remains from la Chapelle-aux-Saints and la Ferrassie (le Museo de l'homme, Paris) and Upper Layer D of Shanidar Cave (Iraq), dated at 40,000 to 54,000 years before present^{1,2}, through the auspices of Professor J. K. Heim. One-third of individuals from la Chapelle-aux-Saints and 17% of individuals from Iraq (Shanidar) had CPPD. We found indentation of the distal radius (wrist) and tibia (ankle) with partial reflection onto the articular surfaces (see figure) in la Chapelle-aux-Saints and Shanidar I Neanderthals, respectively, as well as proximal humeral sub-chondral concretions in Shanidar I specimens and subtle proximal phalangeal smudged



Distal radius articular surface of la Chapelle-aux-Saints Neanderthal viewed with oblique lighting. Peripheral ridge and overhanging edge (partial reflection onto articular surface) are highlighted by their shadow (arrow).

marginal erosions in those from la Chapelle-aux-Saints.

These alterations in shoulder, wrist and ankle are characteristic of CPPD³⁻⁵. The 'smudged' appearance of the proximal interphalangeal joint is similar to that reported in contemporary humans⁶. The ill-defined boundaries of these erosions were easily distinguished from the sharply defined erosions characteristic of rheumatoid arthritis or those associated with reactive new bone formation characteristic of spondyloarthropathy³⁻⁵.

The significance of our identification of these two groups of Neanderthals afflicted by the same disorder prompts some intriguing speculation. If the disease was familial in Neanderthals (as indeed it often is in people today), Trinkaus' hypothesis² that the Shanidar remains were more closely related to "other Near Eastern Neanderthals and more distally related to the European Neanderthals" might be called into question.

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Scientific Correspondence

Scientific Correspondence is intended to provide a forum in which readers may raise points of a scientific character. They need not arise out of anything published in *Nature*. In any case, priority will be given to letters of fewer than 500 words and five references. □

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Voronoi cosmology

SIR—I recently used¹ a simple argument based on analytical results drawn from a paper by Möller² to show that the surprising evidence for periodicity in the line-of-sight distributions from large-galaxy redshift surveys³ is easily explained if the galaxy distribution forms a simple random cellular pattern called a Voronoi tessellation.

Unfortunately, one of the results (equation 7.15) in ref. 2 is not correct. This mistake leads to an error of a factor of 2 in equation (2) of my paper¹. The correct expression should read:

$$\lambda_1 = (81/32n\pi)^{1/3} / \Gamma(2/3)$$

where n is the mean number density of Voronoi nuclei (this revised expression is consistent with the result of an alternative method of calculation⁴). For the preferred value of n , the mean spacing should thus be $69 h^{-1}$ Mpc, not $137 h^{-1}$ Mpc as I originally stated. One would therefore need a much lower value of n to reproduce the observed mean spacing of $\sim 128 h^{-1}$ Mpc (ref. 3) directly; and such a value of n would generate an unacceptably large cluster–cluster correlation length. There is no longer such a nice correspondence between the correlation function of rich clusters of galaxies and the scale of periodicity in the pencil-beam survey as I originally thought, so the motivation for a Voronoi explanation seems to be weakened.

On the other hand, Monte-Carlo simulations of Voronoi universes⁵ show that about 15% of lines-of-sight (consistent with my estimate¹) produce periodicity, but with a spacing of $\sim 107 h^{-1}$ Mpc for the favoured value of n . At first sight this does not look consistent with the revised calculation above, but in the light of my argument¹ that it is the larger Voronoi cells that look regular, selecting a periodic line-of-sight will necessarily select a set of above-average spacings. Taking into account this effect, there seems to be no problem in producing the correct periodicity with an acceptable value of n .

My estimates of the sizes of 'voids', 'great walls' and the estimated probability of observing periodicities are all unaffected, so the Voronoi model still provides a good explanation of how such features can arise from random initial data.

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An omnivorous appetite

Mark Ridley

The Correspondence of Charles Darwin. Volume 6 1856–1857. Edited by F. Burkhardt and S. Smith. Cambridge University Press: 1990. Pp. 673. £35, \$49.50.

TOWARD the end of 1854, Darwin finally completed his eight-year study of barnacles. He was now free to return full-time to the species question. Throughout 1855, he planned, began and accelerated several lines of research on species, and from 1856 to 1857 — the years covered by volume 6 of *The Correspondence of Charles Darwin* — the work was gathering pace.

One large project was on varieties (that is, distinct subspecific forms). Down House became, temporarily, almost a global clearing-house of exotic domestic animals. "I am keeping alive all kinds of domestic pigeons, poultry, ducks", he told his old *Beagle* assistant Syms Covington; and in addition to the live fowls there were the dead ones to be skinned and 'skeletonized'. Darwin's appetite for specimens was omnivorous. On one day, he was after Tumbler pigeons from Walter Elliot in India (they have "the most remarkable inherited habits or instincts": for "when gently shaken and then placed on the ground, the pigeons begin tumbling head over heels"). On another, he was sending to a contact in the Shetlands for a wild rock pigeon. Tributes of fowls, dead and alive, continually poured in from all corners of the globe (often with an awkward postage bill): jungle fowls from West Africa; "Cock & Hen of larger breed, called Lari, from S. Persia"; Jamaican pigeon varieties from a contact of Philip Henry Gosse. Alfred Russel Wallace was collecting for him in the Malaysian archipelago, and the Rajah Brooke of Sarawak sent Borneo fowls ("not of much interest", says Darwin) and cat skins from his imperial domains.

Darwin also had an agent, William Tegetmeier, to work the auctions in London, and traded gifts with fellow fanciers. Thus he tells his son William (whose letters begin "My dear old Willy") how he has received a "present of Trumpeters, Nuns, & Turbits" and has successfully touched "a jolly old brewer" in London for "a pair of pale brown, quite small German pouters". In June 1856, Darwin reported to Tegetmeier that "I counted my pigeons the other day and I have 89" and, more significantly, writes two months later "I am crossing all my varieties to see whether crosses are fertile and for the fun of seeing what sort of creatures appear." That is, he was studying inheritance (not very successfully, it turned out) and was interested in whether any of the varieties had diverged enough not to interbreed (none had). Even without the loss of interbreeding, however, the

pigeons made their point. Charles Lyell visited Darwin at Down in April 1856 and Darwin showed him how the 15 varieties of pigeons he then had differed enough to make "three good genera and about fifteen species according to the received mode of species and genera-making of the best ornithologists."

The meeting with Lyell in April 1856 was an important turning-point. It persuaded Lyell that Darwin was on to something important, and Lyell suggested

arrived by dispersal. He told Hooker "I cannot but think that the theory of continental extension does some little harm by stopping investigation of means of dispersal." It certainly did not stop Darwin, and his experiments on dispersal add recurrent charm to the correspondence. He wrote round his correspondents to collect various types of plant seeds and animal eggs. Several botanists sent him seeds, and snail eggs came from Wollaston (these were particularly important, as Forbes had made a great case out of snails); but lizard's eggs turned out to be more difficult. "I have given up in despair trying to get lizard's eggs in England", he told his cousin William Darwin Fox in October 1856; though in February next year he was still hopeful. We never do find out whether he got any.

Once he had the eggs or seeds, he

IMAGE UNAVAILABLE FOR COPYRIGHT REASONS

Down House, Kent — Darwin filled his home with all manner of natural specimens.

Darwin should put at least an abstract of his views into print. Darwin soon began work on an "abstract"; however (as was the way with Darwin) the mustard seed then started to grow. The abstract was abandoned for a much larger book: the book (to be called *Natural Selection*) that was never completed and was not published even in incomplete form until 1975.

Darwin and Joseph Dalton Hooker (now joined by Asa Gray) continued to discuss geographic distribution. An identical species sometimes lives both on an oceanic island and on the continent some distance away. Older biologists could explain the distribution by "multiple creations" but neither Hooker nor Darwin would have anything to do with that. "Poor E. Forbes" (as Darwin calls him) then suggested the distributions resulted from "continental extensions" that had subsequently sunk. Darwin disliked Forbes' (purely hypothetical) idea, because it required "continental extensions to every island whatever, and that I cannot swallow. Indeed even one continental extension is an awful gulp to me." But Lyell was vexingly sympathetic to the idea, and Hooker remained botanically aloof from the geologist's local squabble; so Darwin was left to protest alone against the sunken continents.

Darwin believed the island species had

immersed them for various periods in sea water to find out whether they could survive. The results were generally excellent. The snails eggs, after long immersion, hatched and "came quite to life and crawled about". Dispersal, Darwin reasoned, could be achieved not only by a seed floating on the sea, but also by one transported inside a bird's guts; and he duly investigated that too: "the hawks [he told Hooker] have behaved like gentlemen & have cast up pellets with lots of seeds in them." Finally, might not seeds be picked up by birds' feet on a muddy day? Darwin's health was too delicate for him to venture out in the rain: Parslow, Darwin's long-suffering butler, was the man for the job. "After heavy rain", one autumnal day, Parslow was evicted from his pantry to shoot partridges; he came back with a brace, but their feet, unsatisfactorily, had "very little dirt". The mail a week or two later brought better material: "I have just received a parcel of partridges feet well caked with mud!!!" What they made of all this in the village we are not told: but there must have been enough stories to have inspired Down's answer to P. G. Wodehouse. □

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The conqueror worm?

Len Goodwin

A History of Human Helminthology. By David Grove. C.A.B. International: 1990. Pp. 848. £55, \$104.50.

WORMS, from mythical dragons to microscopic parasites, have always been a source of fascination. The name itself is an indefinite, emotive and evocative term for "any elongated creeping thing that is not obviously something else". Linnaeus used the title *Vermes* as an all-embracing description for all worm-like creatures: earthworms; insect larvae in fruit, vegetables and trees; parasites in animals and a wide range of other invertebrates. Originally thought to be generated spontaneously in decaying matter and ordure, it took a long time to sort them all out.

When Harold Scott wrote his *History of Tropical Medicine* (Edward Arnold) in 1939 he thought that ankylostomiasis was "almost the only helminthic infestation of man in the tropics which can be said to have a history, at all events a history of sufficient interest to call for any detail" — and he made no mention of any other. He was quite wrong: the stories have all the fascination of good detective yarns. David Grove, of the Department of Clinical Microbiology and Infectious Diseases, Queen Elizabeth Hospital, Woodville, South Australia, who has worked on worm infections in tropical countries, has now written a definitive history of human helminthology. He regards it as a labour of love over the past 12 years, one of them spent as a sabbatical in Britain with access to the data of the *Tropical Diseases Bulletin*, in which the world's helminthological literature has been abstracted since the turn of the century. Detailed and comprehensive, it puts into historical context the discoveries of all those who worked in the field, from the disproof of early theories of spontaneous generation to the systems of classification developed in the eighteenth and nineteenth centuries and the more recent elucidation of life cycles and epidemiology.

There are three chapters of introduction (on classification, general history and the development of anthelmintics), eight on flukes (trematodes), five on tapeworms (cestodes), 11 on roundworms (nematodes) and one on miscellaneous matters such as imaginary worms and pseudoparasites. For each species the discovery of the parasite, its life cycle, clinical manifestations, diagnosis, treatment, epidemiology, preventive and control measures are all covered. Every chapter has a long, valuable list of references to original publications. Writing in a clear,

direct narrative style, Grove retains the reader's attention and interest, even in the more involved arguments and disputes — some of them acrimonious and refreshingly robust — that took place between the holders of rival theories until someone did the right experiment to settle the matter.

A *History of Human Helminthology* is full of good stories. Detailed descriptions are given of the ways in which a guinea worm may be removed, by winding it out on a stick or by snicking the skin at a point half way along and lifting it out with a hook. And there is the intriguing series of disputes on the mode of transmission of Japanese schistosomiasis, settled conclusively by Fujinami in 1909 by showing that cattle, if allowed to walk in the rice paddies acquired heavy infections but did not do so if protected with Wellington boots. The fortitude and devotion to scientific discovery of the early field workers is also impressive. When Arthur Looss (a German with a dogmatic, acrid, controversial style) was working in Cairo at the turn of

the century, the treatment for hookworm disease was unpleasant and uncertain. But having noticed that burning and redness occurred when he accidentally spilt a suspension of hookworm larvae on his skin, he repeated the process, showed that the larvae disappeared and that later, the numbers of hookworm eggs in his stools increased. This was momentous because it was the first time that the percutaneous penetration of any worm was shown.

The book ends with the potted biographies of some 130 prominent personalities in the field, together with their portraits. This helps to bring the text alive — it makes all the difference to know what the actors in the drama looked like. No detailed knowledge of helminthology is assumed and the stories can be enjoyed by all. A splendid achievement, certain to become the standard text on the history of human helminthology for many years. □

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The joy of systematics

Thomas D. Kocher

Molecular Systematics. Edited by David M. Hillis and Craig Moritz. Sinauer: 1990. Pp. 588. Hbk £52, \$65; pbk £29.95, \$37.95.

NEW techniques arise with alarming frequency in molecular systematics. With so many options, it is difficult for novices to determine which method to use for a particular problem. Too often the choice is made not on the merits of a particular approach but by more practical considerations: Dr know-it-all is already using the procedure down the hall. Thus a book that accomplishes a rational evaluation of currently used techniques, as this one does, is most welcome.

As a compendium of classical techniques in molecular systematics, this book will have enduring value. Introductory chapters describe sampling design and give practical details of specimen collection in the field. At the core of the book are six chapters covering the basic methodologies of molecular systematics: isozyme electrophoresis, immunology, cytogenetics, DNA hybridization, restriction fragment analysis and nucleic-acid sequencing. Careful guidance by the editors has resulted in chapters with a uniform appearance and content. Extensive use of figures helps to make the book very readable, despite its encyclopaedic nature. The clear presentation of practical details in experimental protocols makes the book immediately useful in the laboratory. Two final chapters describe the analysis of

data. The chapter by E.L. Swofford and G.J. Olsen on the various algorithms for estimating phylogenies is a real jewel. Each method, and its assumptions, are clearly laid out with a good mix of text, equations and figures. It serves well as a text for graduate-level lectures in phylogenetic reconstruction. Weir's chapter on the analysis of intraspecific differentiation is less successful. Its elegant content will remain inaccessible to most readers. A final chapter by the editors makes recommendations on the choice of techniques for a particular problem.

If the book has a flaw, it is the absence of chapters on molecular evolution. For too long, systematists have proceeded as if the field of molecular evolution had nothing to do with the problems of molecular systematics. Continued emphasis on techniques and algorithms, rather than on a mechanistic understanding of molecular function and the pattern of nucleotide replacement, has led to errors in the interpretation of phylogenies from molecular sequences. Nowhere in this book is the considerable literature on these topics reviewed. Nor is there guidance on choosing nucleotide characters for analysis. These omissions make the book more retrospective than prescient. Increasingly, molecular systematists are finding the direct determination of DNA sequences a practical alternative to more indirect methods of measuring genetic difference. To estimate phylogenies accurately from these nucleotide sequences will require a more complete understanding of evolution at the molecular level than this book can provide. □

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Turbulent affair

Tomas Bohr

Nonlinear Waves, Solitons and Chaos. By Eryk Infeld and George Rowlands. Cambridge University Press: 1990. Pp. 423. Hbk £45, \$85; pbk £17.50, \$29.95.

NONLINEAR waves, solitons, instabilities, singular perturbation methods, inverse scattering, breathers, Bäcklund transformations, Landau damping, chaos, strange attractors, turbulence . . . all exciting subjects about which many of us would like to learn more. These, and a lot more, are covered in this new book by physicists Eryk Infeld from the Institute for Nuclear Studies in Warsaw and George Rowlands of Warwick University. They have collaborated on nonlinear problems for many years and in this book they set out to give a coherent picture of this vast field and how it came about.

The title, *Nonlinear Waves, Solitons and Chaos*, should perhaps have been 'Analytical Methods in the Theory of Nonlinear Waves, Solitons and Chaos'. The authors are clearly fascinated by the different mathematical techniques available (to which they themselves have contributed) and discuss the nonlinear Schrödinger equation, the Korteweg-de Vries equation and the Kadomtsev-Petviashvili equation as if they were old friends (or maybe, faithful patients on which new types of medicine are constantly tried out!). For example, in the chapter titled 'Model Equations and Weakly Nonlinear Theory', it is stated that: "Study of the dynamics of deep water gravity waves has led scientists to a better understanding of the role played by the NLS in weak amplitude theory". Having come this far, the reader of course knows that the NLS is none other than the Schrödinger equation with a cubic nonlinearity and, clearly, the relation between the exact (and very nasty) equations for gravity waves and the NLS is closer to the foreground than the actual physics of gravity waves. This is not to say that the physics is absent, but the stress is on the techniques, which can also be seen from section headings like 'Mystery of the Missing Term' or 'Whitham I'.

Reading the book is a hard, to a large extent, but rewarding work. I especially liked the sections on linear waves and instabilities. The use of Laplace rather than Fourier transforms stresses the boundary conditions and makes, for example, the distinction between convective and absolute instabilities clearer than is usual in even the best textbooks. To aid the reader the discussion of Landau damping might have been supplemented by a drawing of the complex plane with the famous Landau contour. I would also



Jumping for joy — a wild dusky dolphin cavorting off the coast of Kaikoura, New Zealand. While a few countries like New Zealand have laws to protect dolphins, throughout most of the world they are still indiscriminately slaughtered. Taken from *Dolphins: Their Life and Survival* by Michael Donoghue and Annie Wheeler published by Blandford Press at £14.95, and Sheridan House at \$24.95. □

have welcomed some more discussion on the physical origin of the various instabilities — particularly in the chapter 'Solid-Liquid Interface Instabilities' which stands quite alone.

The back of the book deals with approximate techniques and exact methods for a variety of nonlinear partial differential equations are dealt with mainly in the context of surface gravity waves and plasma waves — either in the form of field equations or via the Vlasov equations. I think the uninitiated will need to consult other works both for help through the sometimes rather quick derivations and to understand better the motivation behind various transformations, especially when dealing with exact methods. For example, the section on inverse scattering will probably be hard to follow for a reader who has never seen the Gelfand-Levitan-Marchenko integral equation before. Luckily the authors give relevant references in abundance (sometimes to the extent that the style becomes almost encyclopaedic).

The chapter on chaos contains a description of chaotic maps, period doubling, Poincaré sections and strange attractors, serving as a nice introduction to the subject. One misgiving I have is over the use of the word 'turbulence', which I find confusing. In my opinion the chaos of, for example, the Lorenz equations, or other flows and maps giving low-dimensional

strange attractors, should not be called turbulence. This word should be reserved for systems that are large enough to have chaotic states with both spatial and temporal disorder. Which brings me to my final point: the last chapter seems quite disconnected from the rest of the book. What is needed is a discussion of the very interesting turbulent (that is, chaotic and spatially incoherent) states found in a host of nonlinear equations like the Kuramoto-Sivashinsky equation, the complex Ginzburg-Landau, the nonlinear Schrödinger equation or the Sine-Gordon equation (the latter two including additional non-integrable terms) which the authors know and love. In many of these systems nonlinear 'defects' like solitons, kinks or vortices play an important role in forming the turbulent states and this should make them very interesting to readers of the earlier chapters. Maybe the reason for not including a discussion of these exciting developments is that they are mostly based on computer simulations with somewhat meagre analytical support, and thus differing in style from the rest of the book. Or maybe the authors will be saving these delights for a second volume? Let us hope so. □

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Full steam ahead

Michael Cannon

The Ribosome: Structure, Function and Evolution. Edited by W. E. Hill, A. Dahlberg, R. A. Garrett, P. B. Moore, D. Schlessinger and J. R. Warner. *American Society for Microbiology*: 1990. Pp. 678. \$99.

THE science of the ribosome — a remarkable organelle — covers a period spanning approximately 35 years. *The Ribosome: Structure, Function and Evolution* originates mainly from the presentations of a meeting held in 1989 in the scenic East Glacier Park, Montana. The location of the meeting and the end result are each splendid in their own right.

Peter Moore provides a perceptive introduction to the volume and succeeds admirably in his brief to capture the spirit of the meeting and to summarize the state of the field. After its golden age in the 1960s, ribosome research entered a somewhat less exciting and productive phase and fell out of favour with many in the scientific community and, possibly, with many in the various grant-awarding bodies. In more recent years the pendulum has swung back and interest in the ribosome has revived. This change has resulted, at least in part, from the discovery of RNA enzymes and the development of cloning and sequencing techniques for studying ribosomal RNA. Unfortunately, however, these trends have persuaded some ribosomologists that RNA is all important. Ribosomal proteins hardly have a look in. Crucial though RNA undoubtedly is in determining ribosomal functions, I was pleased to note that Peter Moore

makes a plea for a more balanced outlook. It is to be hoped that more enzymologists join our ranks to unravel additional complexities associated with protein synthesis and its control.

Following Moore's introduction, two distinguished ribosomologists — Masayasu Nomura and Alexander Spirin — provide the reader with their personal views of the history of the study of ribosomes. Nomura treats us to an exciting resumé which brings out his own tremendous enthusiasm for the subject. His fine essay, which describes the key discoveries made over the past three-plus decades, will be of particular interest and value to those who are relatively new to the field. It also provides a walk down Memory Lane for those of us who are, to use Moore's phrase, 'veterans of earlier conventions of the Amalgamated Ribosome Workers'. Spirin covers progress in ribosome preparation, and considers the development of cell-free protein-synthesizing systems. These approaches have been absolutely crucial in aiding our understanding of structure-function relationships within ribosomes.

It would be unfair to single out the excellence or otherwise of the remaining 55 articles — a number that equates with

the number of detectable *Escherichia coli* ribosomal proteins. But it was good to see that most of the authors have made a conscientious effort to provide a review of their own research area in addition to highlighting their own experimental achievements. Undoubtedly this will make it easier for those outside the ribosome field to grasp more quickly the overall relevance of the topics in question. I liked too the short introductions to the 10 sections into which the volume is arranged — a very small touch that gives the book more presentational style.

Meetings such as the one from which this volume derives cannot, of course, materialize without a great deal of input from many people. Furthermore, it is always difficult to decide who should and who should not be asked to contribute a chapter to publications covering scientific conventions. Few could reasonably quibble with the present line up. It is good to see that after wallowing in the doldrums for a little time the ribosome has emerged unscathed and under full steam again. □

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Reading pictures

Richard Gregory

Visual Allusions: Pictures of Perception. By Nicholas Wade. *Erlbaum*: 1990. Pp. 288. £24.95, \$42.95.

VISUAL Allusions is an extended essay by a psychologist-artist. It is not exactly on illusions; but rather, as its title cleverly conveys, on graphical and literary meanings — allusions — of pictures. It is densely illustrated mainly with the author's own photographically produced pictures which have an original quality of deliberate confusion, allusion — and yes — illusion. As Wade says, the text is written round the pictures, and the pictures are to some extent self-explanatory. Several have quite dramatic evocative three-dimensional 'allusions'.

The artist most cogently discussed is Magritte, with new variations on his famous painting of a tobacco pipe. Such questions are raised as: What essential features does the image share with the object? What are the characteristics that correspond to our recognizing certain shapes as representing a pipe? The author concludes that: "Clearly, our recognition of pictorial images can survive all manner of graphical insults!" It is indeed interesting to discover just what can, and what cannot, be changed for object or picture recognition to survive. No doubt this has implications to medical and scientific art,

and interpretations of all manner of pictures and displays, from astronomy to the atom, with or without computer graphics. Verbal descriptions can have profound effects on seeing pictures having scientific content.

Nicholas Wade says (page 145): "Whenever we look at pictorial images we are alluding to features they do not contain." And, "The pictorial art of the 20th century can be thought of as reflecting a concern with graphical image processing." It is not clear, he suggests, which aspects, or features or transformations should be adopted by artists (or scientists?) for their ends. This must mean that the new techniques of image processing offer more possibilities, and so more freedom of expression for artists; which surely is pure gain, though they have to accept that art history is no longer such a valid guide. The snag comes when traditional conventions are not adequate for interpretation. Then discipline and caution, even with special learning required for the viewer, may be needed to 'read' pictures — of art or science.

This brings out the creative role of the observer. It raises questions of how perceptions are related to conceptions. There may not be specific answers here; but the questions are important, and they are quite powerfully evoked by pictures and words. □

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New in paperback

■ From IRL at Oxford University Press come two new books in the Practical Approach Series. *Peptide Hormone Secretion* edited by J. C. Hutton and K. Siddle describes methods for the study of the structure and function of polypeptide hormones. *Postimplantation Mammalian Embryos* edited by A. J. Copp and D. L. Cockcroft provides a detailed introduction to techniques which overcome the problems of embryo inaccessibility, and is aimed at researchers, technicians and students. Both books are priced at £25 (hbk, £35).

■ Out now is the new edition of the *Concise Science Dictionary* from Oxford University Press, which should be of use to all scientists. Price is £7.95.

■ *The Orchids: Natural History and Classification* by Robert L. Dressler provides a modern synthesis on the natural history of orchids. Published by Harvard University Press, price \$19.25, £12.75.

■ Pergamon Press has recently published the second volume of *Progress in Heterocyclic Chemistry* edited by H. Suschitzky and E. F. V. Scriven highlighting recent advances in the field. Price is £21, \$35 (also available in hardback; price £42, \$70). □

Man-made antibodies

Greg Winter & César Milstein

Monoclonal antibodies can now be genetically engineered and endowed with new properties. In the future, gene technology could enable antigen-binding fragments to be made by exploiting repertoires of variable domain genes derived from immunized animals and expressed in bacteria. How readily can this approach be extended to production of '*in vitro*' repertoires of variable domain genes, and obviate the immunization of animals?

In 1975 a method was described for making cell lines that secrete a single species of antibody (monoclonal antibody) with the desired specificity to antigen¹. The technique—'hybridoma technology'—proved to be general, and a wide range of monoclonal antibodies have been made which bind to protein, carbohydrate, nucleic acids and hapten antigens, and which even have catalytic activities^{2,3}, leading to many practical applications for monoclonal antibodies in research and human health-care⁴⁻⁷ and to patent disputes⁸. The technology has been improved over the years, particularly by the preselection of antigen-binding B cells⁹ and by screening with antigen-coated filters¹⁰.

Hybridoma technology was first extended by somatic cell genetics, which allowed antibody mutants to be selected^{11,12}, their functional properties to be changed by switching heavy chain constant regions¹³ and antibodies to be made with dual specificity¹⁴. Gene technology later revolutionized this potential as antibody genes could now be altered to order. New vistas appeared, reviving the forgotten excitement of the old discipline of immunochemistry of antibodies. Initially antibody genes were taken from hybridomas, cloned into plasmid vectors and expressed as complete antibodies in mammalian cells^{15,16} or as fragments in bacteria¹⁷⁻²⁰. The ready manipulation of the genes by cutting and pasting of restriction fragments, or by site-directed mutagenesis, has allowed the construction of new antibody reagents and fine mapping of antibody structure-function relationships.

More recently a new approach has been proposed with the potential to bypass hybridomas^{21,22}. Antibody genes are cloned directly from lymphocytes of immunized animals and expressed in bacteria, and the antibody products are screened for binding to antigen^{23,24}. As does hybridoma technology, the process relies on animal immunization to give rise to many antigen-specific cells. In the animal, antibodies of low affinity are first produced by antigen-induced proliferation of cells, and then higher affinity variants are generated by point mutation and selection. Hybridoma technology can immortalize these cells; gene technology can immortalize their genes. In both cases, however, it is animals that 'invent' the new molecules.

Looking ahead, can we even bypass animals and make new antibodies *in vitro*²⁵? Two current strategies recapitulate the great immunological controversy of the 1950s—instruction versus selection²⁶. The selectionists are making naive repertoires of antibody genes and selecting those with antigen-binding activity, so mimicking nature. The modern instructionists are using computer graphic techniques to build specific antigen-binding sites. Here we shall describe the manipulations that have already produced some designer antibodies of practical value, and discuss other possibilities for improving on nature.

Starting with hybridomas

The antibody (IgG) is a Y-shaped molecule, in which the domains forming the tips of the arms bind to antigen and those forming the stem are responsible for triggering effector functions that eliminate the antigen. The domain structure of the molecule makes it particularly accessible to protein engineering, as func-

tional domains carrying antigen-binding activities (Fv, Fab fragments) or effector functions (Fc fragments) can be used separately as fragments, or swapped between antibodies^{27,28} (see Fig. 1 and legend). Furthermore, the rigid β -sheet framework structure of the variable (V) domains, surmounted with antigen-binding loops, allows the transplanting of binding sites from one antibody to another²⁹. These structural features have spawned a range of designer antibodies, from complete antibodies, capable of destroying pathogens or tumour cells through their natural effector mechanisms, to antigen-binding fragments that can be used to target attached toxins, or for diagnosis though bound radioisotopes (Fig. 2).

Antibodies kill cells by triggering the complement cascade at the cell surface, with consequent lysis, or by binding to receptors on the surface of specialized effector cells, such as phagocytes or killer cells and triggering phagocytosis or antibody-dependent cell-mediated cytotoxicity. The potential of an antibody in lysis is determined mainly by the class of the constant (C) domains (isotype). This has been dissected by making chimaeric antibodies, in which the variable domains of a rodent antibody are attached to the constant domains of human γ isotypes. The human $\gamma 1$ isotype emerges as being highly effective in both complement and cell-mediated killing, and therefore the most suitable for therapeutic use against pathogens or tumour cells^{30,31}. Conversely, the inactive human $\gamma 4$ isotype may be more suitable for diagnostic imaging, or for blocking a damaging immune or allergic response by binding to the antigen and thus competing with destructive antibodies³². Analysis of the roles of individual amino-acid residues should permit the engineering of variants of a single antibody isotype with differing effector mechanisms. Thus the binding sites for the high-affinity receptor (FcRI) include the lower hinge region of the antibody³³, and the 'core' binding site for the first component of complement is a strand of β sheet in the second heavy chain constant (CH2) domain³⁴ (Fig. 1).

Although some rodent antibodies (particularly the mouse $\gamma 2a$ and rat $\gamma 2b$ isotypes), can trigger human effector mechanisms, they are immunogenic in human therapy. In view of the difficulties of making human monoclonal antibodies directly (see later), rodent antibodies have been 'humanized' by linking rodent variable regions and human constant regions²⁸ (chimaeric antibodies, Fig. 2). This reduces the immunogenicity of the antibody as shown in clinical trials³⁵. But residual immunogenicity is retained (at least in part) by virtue of the foreign V-region framework³⁶.

A more complete way of humanizing rodent antibodies includes the replacement of the V-region framework ('reshaped' antibodies, Fig. 2). It relies on the architecture of V domains as a framework of β sheets topped with antigen-binding loops (see ref. 37 and Fig. 1). By grafting the loops, the antigen-binding site can be transferred from rodent to human antibody^{29,31,38,39}. The technique was used to humanize a rat therapeutic antibody directed against mature human leukocytes³¹ which proved clinically effective in destroying a large mass of tumour in two patients⁴⁰. But reshaping requires that the different framework

regions are structurally conserved, both in the orientations of the two β sheets of each domain and in the packing of heavy chain variable (VH) and light chain variable (VL) domains together; that the hypervariable loops make the majority of contacts with antigen; and that the loops are supported in a similar way by the underlying β -sheet framework. Although these are likely to be true for some antibodies, the restitution of key contacts between loops and framework has proved

necessary in others. Simple molecular modelling can help identify contacts and design small changes to optimize affinities^{31,41}.

Although natural effector functions are powerful, antibodies can also be engineered to recruit other effector functions. For example, by generating antibodies whose antigen-binding sites have dual specificity for a target cell and an effector such as a toxin. Such 'hybrid hybridomas' are made by fusion of hybridomas of two-different specificities^{14,42}, each of the two

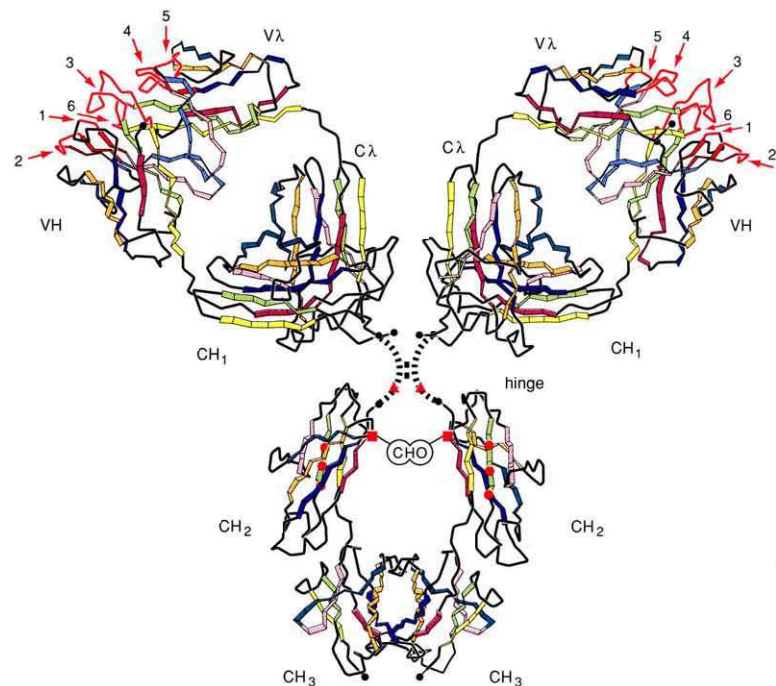
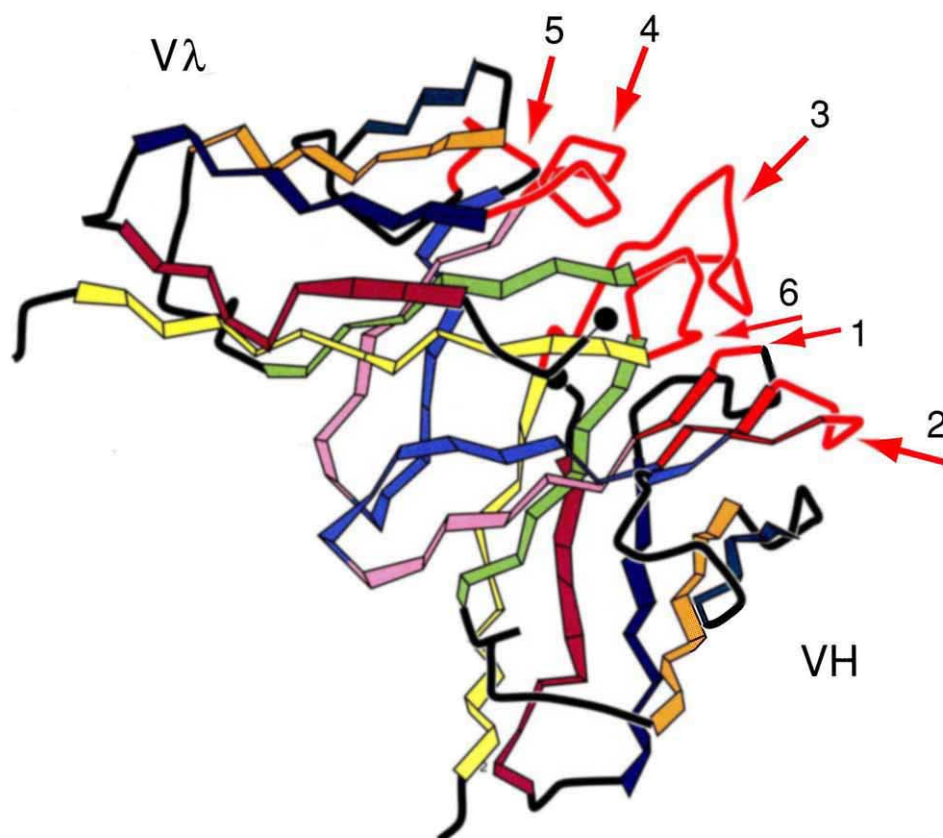


FIG. 1 Antibody structure. The antibody (IgG) consists of four chains, two heavy (H) and two light (L), which in turn are built by stringing together domains of similar architecture. Each chain is paired with another chain by lateral packing of the domains and also by at least one disulphide bond. Each domain consists of two β sheets which pack together to form a sandwich, with exposed loops at the ends of the strands. Thus the C domains have three β strands in one sheet (strands C, F, G) and four strands (strands A, B, D, E) in the other. The V domains have an extra two strands in one sheet (C, C', F, G). This framework is highly conserved in different antibodies. The three loops at the top of the V domain are hypervariable in sequence and fashion the antigen-binding site³⁷. Despite the sequence hypervariability, most of the antigen-binding loops have a small repertoire of main-chain conformations. The antibody can be proteolytically cleaved at the flexible hinge region, yielding Fab fragments which bind antigen (comprising heavy chain VH and CH1 domains, top of the hinge and the entire light chain), and Fc fragments which bind to effector functions (comprising lower hinge and heavy chain CH2 and CH3 domains). The model is taken from the solved X-ray crystallographic structures of Fab and Fc domains of myeloma protein KOL⁸⁵, represented according to Lesk⁸⁶ and redrawn by an artist. Each strand of the β sheets has been colour-coded. The β sheet defined by the A, B, D, E strands is more intensely coloured. The hypervariable regions (VH, 1-3; VL, 4-6) are in red; the binding sites for high-affinity receptor³³, C1q³⁴ and carbohydrate attachment site are also marked in red, as triangles, circles or squares respectively.



halves of a single antibody molecule is from one of the two parental antibodies, and the molecule therefore carries two different antigen-binding sites. Such bispecific antibodies can bind both to a cell target (for example in a tumour) and to a toxin or cytotoxic T cell^{43,44}. Novel effector functions can also be recruited by fusing gene segments encoding antigen-binding sites (as Fc or Fab fragments) to genes encoding toxins⁴⁵ and enzymes²⁷. This can, for example, allow agents such as tissue plasminogen activator to be targeted to blood clots through Fab binding to fibrin, leading to the local activation of plasminogen⁴⁶. Fusion of antibodies with enzymes may also prove invaluable for local activation of prodrugs^{7,47}. Conversely antibody Fc fragments can equip other proteins with antibody effector functions. For example, CD4 linked to Fc fragments binds to viral glycoprotein gp120 on the surface of cells infected with human immunodeficiency virus (HIV), and kills the cells by antibody-dependent cell-mediated cytotoxicity (ref. 48) (CD4 immunoadhesin, Fig. 2).

Complete antibodies (relative molecular mass 150,000 (M_r 150K)) are large molecules but much smaller fragments can be prepared that retain antigen-binding activity. Small fragments (M_r 12K–50K) equipped with radioisotopes could be used for imaging or therapy and are in some ways particularly attractive for use *in vivo* as they penetrate tissue boundaries more effectively⁴⁹. Fragments are also cleared faster from the serum

and tissues⁵⁰ and although this may compromise their use as targeting agents, it can aid the clearance of toxic drugs, such as digoxin, from the circulation⁵¹. Small fragments also have advantages in fundamental research, for high-resolution X-ray crystallographic studies of antigen-binding sites. The size of antibodies and flexibility of the hinge connecting Fab arms and the Fc domain have prompted crystallographers to turn to Fab fragments⁵² and now Fv fragments⁵³ (Fig. 2). In future we expect that fragments will be used extensively as they are readily expressed in an active form from genes introduced into mammalian^{27,54} or bacterial cells^{19,20,23,55}. But antibody fragments may require further engineering to eliminate undesirable properties.

For example, Fv fragments are noncovalently associated heterodimers of VH and VL domains which may thus dissociate. Although some Fv fragments are less prone to dissociation than others⁵⁶, stable Fv fragments can be engineered either by linking the domains with a hydrophilic and flexible peptide^{57,58} to create single-chain Fv fragments (scFv, Fig. 2), or by introducing disulphide bonds between the domains⁵⁶. Single VH domains (dAb, Fig. 2) with antigen-binding activities²³ are likely to require more extensive engineering. The domains have an exposed hydrophobic surface (where they normally interact with light chain), rendering them 'sticky'. If their properties can be improved, for example by introducing hydrophilic residues to the interface, these single domains may prove to be the forerunners of a new generation of small recognition molecules.

Bypassing hybridomas

Immunization is essential to derive hybridomas secreting high-affinity antibodies. In the animal these antibodies are produced in two stages^{59,60}. The first stage is fast and leads to the production of antibodies of low affinity (10^5 – $10^7 M^{-1}$). It involves the

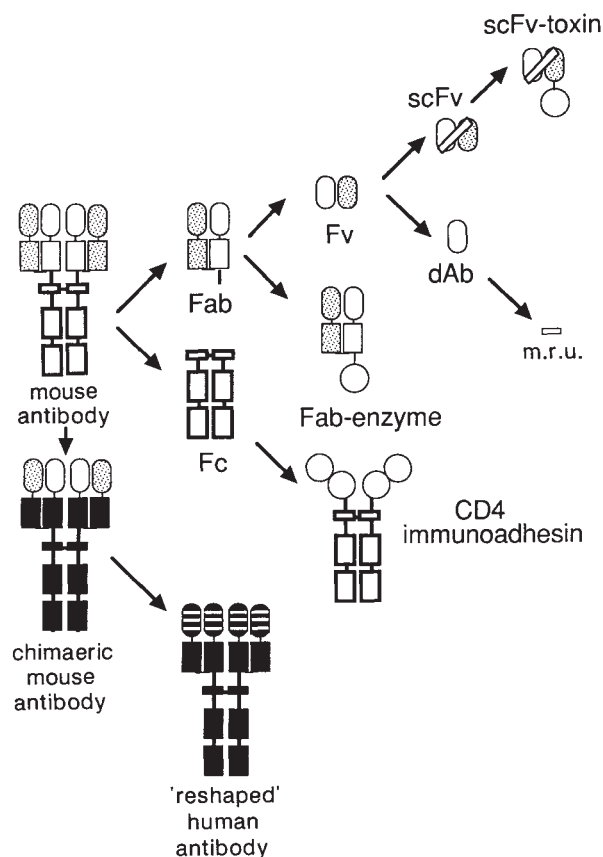


FIG. 2 Engineered antibodies and fragments. A range of engineered antibodies and fragments is depicted. Each box represents a domain. Single-chain Fv fragments (scFv), in which VH and VL domains are linked by a peptide (see text), and Fab fragments have been used to target enzymes and toxins. In immunoadhesins, a ligand specific for receptor (here CD4) is attached to an Fc fragment. Single VH domains (dAbs)²³ and even single CRRs (minimal recognition units or m.r.u.)^{87,88} have been identified with antigen-binding activities. Site-directed mutagenesis of antibodies and fragments has also been used to alter effector functions and improve affinities⁴¹ (see text).

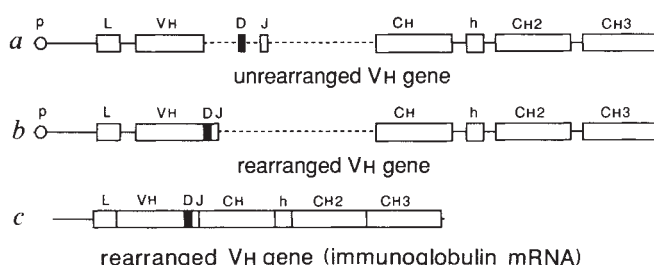


FIG. 3 Organization of V genes. The domain structure of the antibodies is mirrored at the level of the gene, as the individual domains are encoded by separate exons. In turn, the VH and VL domains are built from separate genetic elements, and assembled by DNA rearrangements during lymphocyte differentiation. Thus the first and second hypervariable loops are encoded by the germ-line V genes, but the third hypervariable loop by the combination of V, D and J elements (for the VH domain) and V and J elements (for the VL domain). The third hypervariable loop of VH is accordingly the most diverse in sequence and backbone conformation and is often the longest of the loops. Exon structure is illustrated for the unrearranged and rearranged VH genes and is similar (not shown) for VL genes (VK or VL). VH, unrearranged or rearranged heavy chain variable region as appropriate; D, D segment; J, J segment; CH1, CH2, CH3, first, second and third heavy chain constant exons, respectively; h, hinge exon; L, signal sequence; p, immunoglobulin promoter (octanucleotide motif). The potential diversity of the mouse primary repertoire is huge as a consequence of the combinatorial arrangements of the genetic elements. It can be estimated as follows. (1) Diversity due to combinatorial integration: $300 V_K \times 4 J_K = 1.2 \times 10^3$ and $200 V_H \times 15 D \times 4 J = 12 \times 10^3$. (2) Diversity due to junctional alternatives (alternative readings at junction due to differences in reading frame, and lengths of J and D segments): V_K to $J_K \sim 3$ and V_H to D to $J \sim 40$. (3) Diversity due to N segment for heavy chain >10 . (4) Hence combined diversity for each chain: light chains $= 3 \times 10^3$ and heavy chains $> 5 \times 10^6$. Conclusion: diversity due to combinatorial association of heavy and light chains $> 10^{10}$. Additional factor due to somatic mutation $> 10^{30}$ (see text).

TABLE 1 Immortalization of antibodies using unselected cells from hyperimmunized animals

	Hybridomas	EBV transformation	Random combinatorial gene cloning (bacteria)
Species restrictions	Yes	Only human	No
Chain pairs	H and L	H and L	H and L separate
Positive/negative clones	1/50	1/500(?)	<1/10 ⁴ ('artificial')* <1/10 ⁸ ('original')
Assay on plates (number of clones as replicas or overlays per plate)	~300	ND	~10 ³⁻⁵ (?)
Assays on supernatants	Well established, very sensitive	Well established, less sensitive	Possible
Doubling time of cells	18 h	~40 h	30 min
Stability	Good	Poor	Very good
Product	Full molecules	Full molecules	Fragments
Yield of product	20–100 µg ml ⁻¹	0.5–5 µg ml ⁻¹	0.5–10 µg ml ⁻¹

* These values are calculated as follows. If P is probability of finding a particular combination of light (L) and heavy (H) chains in an N -size combinatorial library, and P_L and P_H are the probabilities of random occurrence of each component, $P = 1 - e^{-q}$ where q is $NP_L P_H$. If we assume that 1% of the mRNA molecules encode antigen-specific immunoglobulin ($P_L = P_H = 10^{-2}$), there is ~60% probability that 1/10⁴ recombinants will contain one H and one L chain encoding any antigen-specific immunoglobulin: most are comprised of artificial combinations, which may or may not specify antigen-binding activity. If there is a dominant lymphocyte clone which represents 1% of the antigen-specific immunoglobulin lymphocytes, the frequency of each chain is 10⁻⁴ and testing 10⁸ clones will give us a 60% probability of finding the 'original' combination. But regardless of whether they originate from antigen-binding lymphocytes, L chains may be capable of complementing the H chains from antigen-specific immunoglobulin. This frequency may be high particularly in primary responses and early secondary responses, before extensive diversification through hypermutation, but will depend on the cut-off affinity used in the assay. Taking a value of 1:100 (ref. 69), there is a 60% chance that ~10⁴ of the recombinants will give an 'artificial' positive signal. EBV, Epstein-Barr virus.

proliferation of cells drawn from the repertoire already available at the time of immunization. The potential repertoire is huge (>10¹⁰) (Fig. 3 and legend), but at any given time, only a fraction of the potential repertoire of a mouse is available through the limited number of clones (10⁷–10⁸) expressing antibodies.

The second stage produces high-affinity antibodies, starting with the genes used in the first stage. Its main tool is the hypermutation of these genes followed by selection of those cells which produce antibodies of increased affinity. This is a darwinian process, involving variation resulting from point mutations followed by selection driven by the requirement for antigen for cell survival. It is an inheritable process, but only at the somatic level among the lymphoid cells of the individual. The resulting memory cells seem to be able to undergo new rounds of hypermutation and selection following antigenic challenge⁶¹.

The rate of mutation approaches 10⁻³ or 3 × 10⁻⁴ mutations per base pair per cell division in a more recent estimate^{60,61,62}. Mutations are localized to the segment coding for the variable portion of the antibody genes. The potential diversity generated by hypermutation is astronomical. Even if only the hypervariable region (~30 residues) of a single antibody variable domain could mutate into 10 out of the 20 amino acids, this would produce about 10³⁰ variants for each chain. So the problem at this stage is not the generation of diversity, but the continuous selection of improvements against a background of degeneracy⁶³. As immunization proceeds, additional high-affinity antibodies gradually emerge with V–D–J combinations which are rarely found in the primary repertoire (repertoire shift)⁶⁴.

In 'classical' antibody engineering, hybridomas of known specificity have provided the raw material for cloning the rearranged antibody VH and VL genes. However, using 'universal' primers and the polymerase chain reaction (PCR) it is possible to rescue V genes^{21,23,65,66} and by building restriction sites into these primers the amplified DNA can be cloned directly for expression in mammalian cells²¹ or bacteria^{23,24}.

This offers new routes for the derivation of monoclonal antibody-producing cell lines (Fig. 5). At its simplest, it allows V genes to be rescued from hybridomas²¹, unstable hybridoma fusions (for example mouse-human hybridomas), single hybridoma cells^{67,68} or even single B lymphocytes. Hybridomas have advantages because fusion enriches for antigen-stimulated cells. However, rescue from single B lymphocytes bypasses cell fusion, allowing access to terminally differentiated B cells which

are rich in messenger RNA but are not able to fuse. Single hybridomas or B cells might also be isolated by binding to antigen, immobilized on solid supports (for example on plates or magnetic beads), by fluorescence-activated cell sorting (FACS) or by rosetting with antigen-coated red cells. The single set of V genes from individual cells could then be co-expressed in bacteria. But in this case each cell must be processed separately.

Cloning from heterogeneous cell populations has the advantage that all the cells can be processed together. For example,

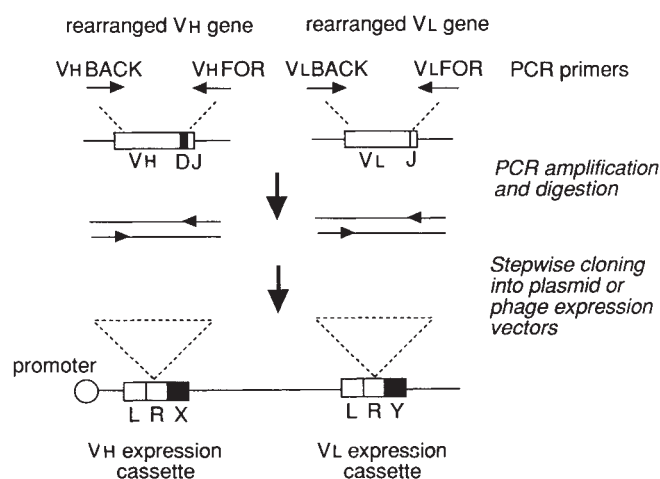


FIG. 4 PCR cloning of rearranged VH and VL genes into expression vectors. Primer mixtures can be designed for PCR amplification of most families of V genes, as the nucleotide sequences at the 5' and 3' ends of the rearranged V genes are relatively conserved²¹. At the 5' end, mixed PCR primers have been based within the signal sequence^{65,67} or at the N-terminal end of the mature variable domain^{21,66}. At the 3' end, PCR primers have been located within the J region or the C region. One set of PCR primers, located entirely within the rearranged V-gene exon, amplifies both mRNA and chromosomal DNA^{21,23}. The PCR primers incorporate restriction sites and after amplification and digestion with restriction enzymes can be cloned into specialized plasmid or λ phage vectors directly for expression of the V genes. The cloning of VH and VL genes into a plasmid expression vector is illustrated here. (L, leader sequence for secretion into bacterial periplasm and/or beyond outer membrane; R, polylinker cloning site; X and Y, extra polypeptide sequence (for example, heavy chain CH1 domain, light chain CL domain or peptide tag for recognition by monoclonal antibody).)

VH and VL genes, taken from total unfractionated cells from an immunized mouse have been combined at random and Fab fragments expressed from λ phage screened for antigen-binding activities²⁴. The disadvantage is that the original VH and VL pairing, selected for high affinity by immunization, is lost. A combinatorial library with only 1,000 different VH and 1,000 different VL gene elements equally represented, would necessitate the screening of between 10^6 and 5×10^6 clones to recover most of the original pairings. Thus the chances of recovering original pairs of V genes from a large random combinatorial library from an immunized mouse are low, and even more remote for the highest affinity antibodies of hyperimmune animals. Those pairs of VH and VL domains that are found to be capable of binding antigen^{69,70} are likely to do so at lower affinity or with lower specificity than those selected by antigen. Although isolation of an anti-hapten Fab fragment with a good affinity has been reported²⁴ using such an approach, if the aim of this technology is to bypass hybridomas, it will need to make the high-affinity antibodies required for diagnosis and therapy, antibodies which are difficult to derive even with hybridomas.

To improve the chances of recovering original pairs, the complexity of the combinatorial libraries could be reduced by using small populations of antigen-selected B lymphocytes (Fig. 5). But all combinatorial approaches will rely heavily on powerful screening methods. Here the use of membrane filters for screening large numbers of clones is promising. A variety of formats have been deployed; for example, capture of antibodies on filters coated with antigen, and detection with anti-globulin reagents¹⁰. Alternatively, antibodies or Fab fragments have been immobilized directly on membrane filters²⁴ or indirectly through antiglobulin reagents (A. Skerra, M. Dreher, E. Gheradi, G.W. and C.M., unpublished results) and probed with labelled antigen.

Bypassing animals?

Looking ahead, it may become possible to build antibodies from first principles, taking advantage of the structural framework on which the antigen-binding loops fold. In general these loops have a limited number of conformations which generate an endless variety of binding sites by alternative combinations and by diverse side chains^{71,72,64}. Recent successes in modelling antigen-binding sites^{41,72} augurs well for *de novo* design. This approach might become attractive for making catalytic antibodies, particularly for small substrates. Here side chains or binding sites for prosthetic groups⁷³ might be introduced, not only to bind selectively to the transition state of the substrate, but also to participate directly in bond making and breaking. The only question is whether the antibody architecture, specialized for binding, is the best starting point for building catalysts. Genuine enzyme architectures, such as the TIM barrel, might be more suitable. Like antibodies, TIM enzymes also have a framework structure (a barrel of β strands and α helices) and loops to bind substrate. Many enzymes with a diversity of catalytic properties are based on this architecture⁷⁴ and the loops might be manipulated independently of the frameworks⁷⁵ for design of new catalytic and binding properties.

Instead of the 'design and build' approach, could we build an artificial selection system, for example harnessing bacteria or phage, to select for antigen-binding activities? Here no structural information about the antigen is needed. But we see no future in trying to select high-affinity binding activities in a single step. The strategy of the immune system, in which low affinity evolves to high affinity seems more realistic²⁵. Can we imitate this strategy and indeed improve on it?

Our first task is to prepare a naive repertoire of antibody genes (Fig. 6). At its simplest we could use the polymerase chain reaction (PCR) and universal primers to reproduce *in vitro* the repertoire of rearranged V genes expressed by naive animals. However 'naive' animals are not really naive and the available repertoire is limited in size and shaped, for example by tolerance

to self-epitopes. In principle we could adopt the mammalian strategy of assembling a much larger repertoire by random combination between restriction fragments encoding the germ-line V, D and J elements. To match the potential repertoire of the animal, we would also have to reproduce the junctional diversity created by recombination. Large repertoires might also be made by trying to imitate the process of gene conversion adopted by birds⁷⁶.

But such 'natural' repertoires are not ideal. The sequences of germ-line V genes and the multiplicity of highly related V genes in the genome are presumably themselves the result of chance and evolutionary pressures, for example towards recognition of pathogens and against recognition of self-components. Such repertoires are both biased and highly redundant. More efficient repertoires might be constructed by using V genes from a variety of animal sources, excluding highly related V genes and even designing entirely new V genes or D segments. For example, the structures of individual antibody loops are often very similar

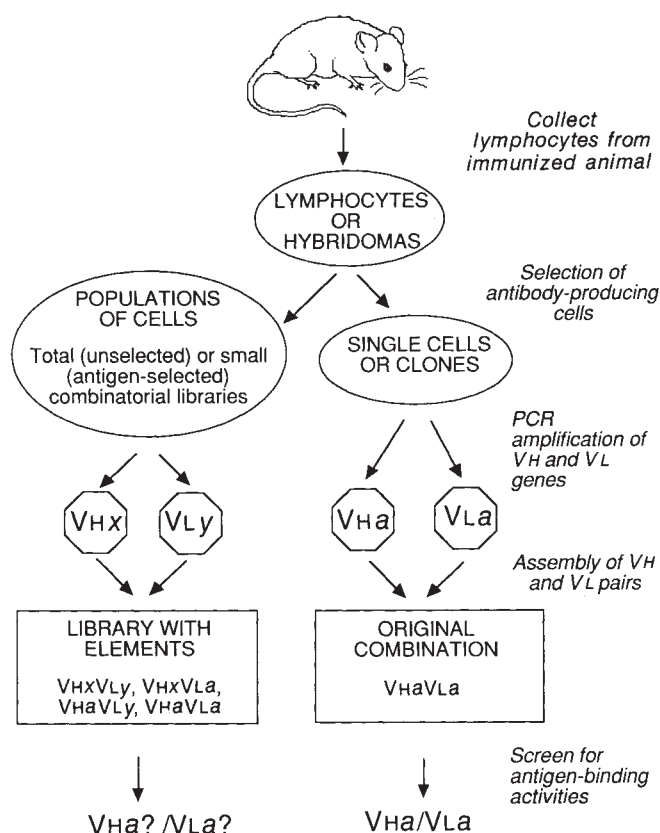


FIG. 5 Strategies for cloning paired VH and VL genes from lymphocytes of an immunized animal. VH and VL genes (expressed as pairs VHx/VLy) from n lymphocytes can be amplified by PCR from the mRNA or genomic DNA of single cells or populations. The lymphocytes can also be selected by antigen-binding, or immortalized as hybridomas by fusion with myeloma cells or by infection with Epstein-Barr virus. From single lymphocytes, or clones, the original VHa/VL α gene combination is readily rescued. From populations of lymphocytes, repertoires of VH-VL genes (VL n) could be combined at random, and antigen-binding combinations selected. But for large populations of lymphocytes, original combinations of VH and VL genes will be a minor proportion of heterologous combinations, some of which may display residual antigen-binding activity. The library will contain $(n-1)^2$ VHxVLy elements, $(n-1)$ each of VHxVL α and VHaVLy elements and a single VHaVL α element. VHa and VL α (or close derivatives), however, may occur repeatedly. Thus, at earlier stages or immunization, there are likely to be some artificial pairs which originate from different but closely related clones which can bind antigen, particularly with responses dominated by 'unique' VH and VL gene combinations (restricted or idiotypic responses^{59,60}). Ref. 90 is in agreement with this prediction. As maturation for high affinity proceeds, the number of mutations increase and the likelihood of effective heterologous complementation diminishes.

in their overall fold^{71,72}, and the loops could be fleshed with diverse side chains.

The next stage is to express the library and screen for antigen-binding activities by random combination of V_H and V_L domains. Ideally the library should be 'complete', containing antibodies of a minimum binding affinity for any conceivable epitope. The tighter the binding of a primary antibody, the larger the library required. For example, it has been estimated that a primary repertoire of 10⁷ different antibodies is likely to recognize more than 99 per cent of epitopes with an affinity constant of 10⁵ M⁻¹ or better, and rarely contributes high-affinity antibodies to an epitope taken at random (>10⁹ M⁻¹) (ref. 77). If millions of V_H and V_L combinations from an artificial library could be screened, for example on membrane filters with a cut-off affinity of at least 10⁵ M⁻¹, then the library would be as complete as the primary repertoire of a single mouse (10⁷ antibody species). In the future, the screening of large libraries may well be replaced by methods of selection. For example, by expressing functional antibody fragments at the surface of phage, desired V-gene combinations can be enriched by binding to antigen⁷⁸. If these screening and selection techniques worked as well as those in the animal, we could imitate the primary response and generally obtain low-affinity antibodies.

The enhancement of affinity *in vivo* can be contributed by a single point mutation, or several mutations⁶⁴. Hypermutation of the genes corresponding to antibodies of low affinity should be easy to imitate *in vitro*. Point mutations could be introduced into the V genes by many techniques, for example, by using error-prone polymerases, PCR amplification through a large number of cycles, biased ratios of nucleotide triphosphates or 'spiked' oligonucleotide primers. Multiple mutations could be targeted throughout the body of the gene simultaneously, or to each of the hypervariable loops⁷⁹.

The screening or selection of the mutants with improved affinity is likely to be more difficult, as it needs to discriminate between mutants differing slightly in affinity. Such discrimination is possible for hybridoma clones in agarose¹⁰, by binding secreted antibody on coated membranes with different antigen density. The differential binding to antigen-coated membranes might also be achieved by competition with low-affinity antibody. The system we are proposing, however, is primitive compared with the animal. Ideally we would like to hypermutate specific segments of DNA within cells rather than in isolated DNA, and at the same time express the products on the cell surface, and select in a darwinian fashion variants of steadily increasing affinity. Animals are superb at this job, and it will not be easy to compete with their efficiency. We may learn to imitate the animal strategy, but in the meantime for the production of high-affinity antibodies we will normally do better by stealing hypermutated V genes from immunized animals.

Human antibodies: today and tomorrow

Making human monoclonal antibodies has posed difficulties for hybridoma technology. For example, the use of the mouse myeloma as a fusion partner for human cells leads to preferential loss of human chromosomes, and intolerable instability of the hybrids. Hybridomas are derived from spleen or lymph nodes, whereas the primary source from humans, the peripheral blood lymphocytes, contains few blast cells actively involved in the immune response. As an alternative to fusion, immortalization of human cells by Epstein-Barr virus does not lead to preferential immortalization of blasts engaged in antibody responses, and also leads to lines which are low producers of antibody and unstable⁸⁰. Further, humans can rarely be hyperimmunized to order, especially with noxious chemicals, pathogenic viruses or cancer cells. In any case, the isolation of human antibodies to human cell-surface antigens would have to overcome tolerance mechanisms which eliminate lymphocytes with self-reactivity. Human lymphocytes have recently been used to populate severe

combined immunodeficient (SCID) mice and these animals can be immunized^{81,82} to make human antibodies.

Gene technology offers alternatives. The 'humanizing' of rodent monoclonal antibodies is currently the most practical approach. It allows access to a vast pool of rodent antibodies with good affinities and specificities. This is a major advantage, particularly when dealing with antibodies for tumour therapy, or for the manipulation *in vivo* of the human immune system. Thousands of antibodies have already been made against human

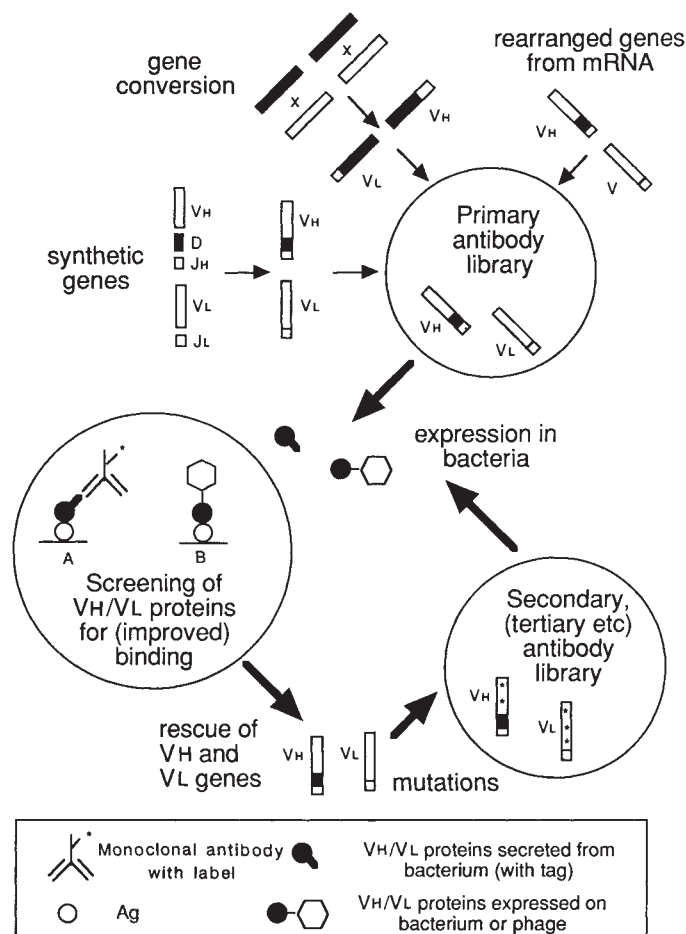


FIG. 6 Mimicking the immune system. V_H and V_L gene repertoires derived by a range of methods, such as reproduction of the available library from lymphoid cells, assembly of V, D and J elements, or gene cross over (to mimic gene conversion). The genes are cloned and expressed, and V_H-V_L pairs binding to antigen can be selected or screened, for example on membrane filters. Here the V_H-V_L pair, binding to antigen (Ag), is tagged with a C-terminal peptide recognized by a monoclonal antibody (mAb), or is expressed on the surface of a bacterium or phage. For detection, the mAb or Ag can, for example, be coupled to radioisotope, or to an enzyme for production of a coloured dye. A, Capture of V_H-V_L with antigen and detection with mAb. Other schemes could involve capture by binding to membrane and detection with Ag or capture by mAb and detection with Ag. B, Capture with Ag and detection of phage plaques. The genes encoding V_H-V_L pairs, identified as antigen-binding, can be rescued and hypermutated to form the secondary antibody library. These genes can then be expressed and screened for improved binding to antigen. This process will need to be repeated several times to achieve the efficiency of animal immune systems. Selection systems for catalytic antibodies might differ from those above. Such antibodies have been made through hybridoma technology by immunization with transition-state analogues, or by chance^{2,3}. But animals select antibodies on the basis of binding, not catalysis, and the transfer of V-gene repertoires to bacteria gives a new twist to the field²⁴. Vast libraries might be screened directly, not for binding, but for catalysis for example with substrates yielding fluorescent products, or by complementation of bacterial mutants deficient in a catalytic step of interest. In this case, antibodies will need to be expressed intracellularly⁸⁹.

cell-surface antigens, and particularly against human leukocytes⁴. Reshaping these antibodies, by transplanting only the antigen-binding loops to human antibodies^{29,31}, yields humanized antibodies which may have similar immunogenicity to truly human antibodies. In future, several other approaches may become available through gene technology as discussed in earlier sections. Furthermore, transgenic mice have been made which carry immunoglobulin heavy V, D, J and human C regions⁸³ and this should allow human antibodies to be produced directly from hyperimmunized mice. It remains to be seen whether the size of the repertoires, limited by the amount of new DNA that can be carried by the transgenic animal, will be a drawback.

But all these methods will have to compete with immortalization by Epstein-Barr virus and cell fusion, which themselves are constantly improving, particularly as they start to incorporate

ideas and techniques involving DNA manipulations. There are immune responses which are unique to humans, or which need to be characterized in humans, including those to antibodies in certain auto-immune, parasitic or infectious diseases, or cancer. Here the rescue by gene technology of human hybridomas or cell lines immortalized by Epstein-Barr virus may provide a powerful way of defining the properties of such antibodies and immortalizing them⁶⁸.

As Ehrlich wrote 90 years ago⁸⁴, '... we have already caught a distinct glimpse of the land which we hope, nay, which we expect, will yield rich treasures for biology and therapeutics'. We see a jungle of technologies, old and new, stimulating each other: in the immediate future, most of them start with immunizing animals. □

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Evidence from balloon measurements for chemical depletion of stratospheric ozone in the Arctic winter of 1989–90

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Measurements of the vertical ozone profile with balloon-borne sensors in the Arctic during January and February 1990 indicate repeated minima in the 22-km region. A general negative correlation of the 22-km ozone mixing ratio with time spent by the air parcel in regions cold enough for the formation of polar stratospheric clouds, together with the presence of sunlight on a portion of the air-parcel trajectory, and an inability to explain the ozone minimum by conventional dynamical processes, suggest that this feature is related to chemical ozone depletion.

THE clear identification of springtime ozone depletion in the Arctic stratosphere, related to elevated levels of chlorine as observed in Antarctica, has yet to be achieved. Following an unusually cold stratosphere in January 1989, balloon-borne measurements of the ozone profile at Kiruna, Sweden, indicated reductions of 10–25% in the altitude range of 22–26 km, which amounted to only ~3% in total ozone¹. From comparisons with Antarctic data under similar solar illumination periods, these minima were interpreted as indicating the beginning of chemical ozone depletion. From aircraft measurements of elevated levels of chlorine monoxide (ClO) in the lower stratosphere in the Arctic in 1989², it was deduced that the region was primed for ozone depletion. Aircraft lidar data in early February 1989 indicated that ozone mixing ratios were ~15% lower at 20 km in the outer vortex, which had been exposed to sunlight, relative to the inner vortex³. Comparisons of ozone and nitrous oxide, measured *in situ*, in the Arctic stratosphere in February 1989, indicated ozone depletions of 12–35% in the vortex relative to its exterior⁴. Although the Arctic polar vortex did not remain intact long enough in 1989 for substantial ozone depletion to take place, and there were no observations of a continual reduction in ozone as is observed in the Antarctic spring^{5,6}, evidence for a lesser degree of chemical ozone depletion existed. An ozone measurement programme, similar to those carried out in Antarctica since 1986, was clearly needed to investigate further the issue of Arctic ozone depletion. Such a measurement campaign was conducted at Esrange (68° N, 21° E), near Kiruna, Sweden, from 8 January to 8 February 1990.

Observations

Ozone profiles were measured with digital electrochemical ozonesondes⁵ on 31 occasions during the one-month measuring period. The air sample pump on each ozonesonde was individually calibrated to pressures of 3 hPa (equivalent to an altitude of ~35 km) because flow-rate corrections at low pressures vary from unit to unit. Although most of the flights reached altitudes of ~30 km, about half reached 32–35 km, allowing Arctic winter ozone profiles at these extreme altitudes to be studied.

Although the 30-hPa temperatures in January did not start

off as cold as in 1989, they reached lows of ~−93 °C on 6 February. As shown in Fig. 1, temperatures in the 20–25-km region over northern Scandinavia were less than −80 °C after 10 January, except for a two-day period from 20 to 22 January. These temperatures are low enough for clouds composed of nitric acid trihydrate (NAT) to form (assuming nominal values of H₂O and HNO₃ mixing ratios of 5 p.p.m.v. and 10 parts per 10⁹ by volume (p.p.b.v.), respectively⁷), and polar stratospheric clouds (PSCs) were indeed detected with balloon-borne sensors on all eight flights after mid-January, on 13, 16, 19, 24, 27, 31 January, and on 2 and 6 February⁸. PSCs were also detected at Alert, North-West Territory (82° N, 61.5° W) during mid-January in 1990⁹. Thus, some degree of chlorine activation by PSCs probably occurred in the Arctic polar vortex during the winter of 1990. The amount of ozone depletion would then be dictated by the time air parcels spend in sunlight, as sunlight is needed to complete the chlorine-catalysed cycle of ozone depletion¹⁰.

Also shown in Fig. 1 is the total column ozone determined for the 31 soundings from integration of the ozone partial pressure profiles. Following low values on the first two soundings, associated with unusually low ozone concentrations below 20 km, the general nature of the total ozone variation is a reduction from an average value of about 340 Dobson Units (DU) on day 9 to an average value of ~300 DU on day 24. Total ozone then fluctuated about a value of ~310 DU until day 38 when a value of 435 DU was observed on the last sounding, corresponding to rapid warming in the stratosphere over Scandinavia. Stratospheric temperature data in Fig. 1 indicate a trend similar to that of total ozone. Temperatures decreased with time at all altitudes between ~10 and 28 km (pressures of 100 and 10 hPa) from day 7 to 24 and then remained relatively constant except for extreme cold periods between 20 and 25 km around day 30 and days 34 to 37. The rapid warming observed on day 38 is not represented in the temperature data in Fig. 1.

From a detailed study of Arctic ozone and temperature data, Bojkov¹¹ showed that a positive correlation existed between total ozone and stratospheric temperature. This correlation is related to ozone transport, whether vertical (adiabatic ascent resulting in cooling and reduced ozone, or subsidence resulting in warming and increased ozone on constant pressure surfaces) or horizontal (advection of warm, ozone-rich air, or cool, ozone-poor air). Bojkov¹¹ also showed clearly that the trend from about November to April is one of increasing ozone at all Arctic stations, owing to advection of ozone-rich air into the polar regions. Data at 30 hPa from the Nimbus-7 limb infrared monitor of the stratosphere (LIMS), for the latitude belt 76°–84° N and the period 25 October 1978 to 28 May 1979¹², indicate an increase in ozone mixing ratio along an isentropic surface of ~0.3 p.p.m.v. per month during diabatic cooling of ~0.5 °C per month, from November 1978 until the major stratospheric warming in mid-January 1979. Thus all available Arctic ozone data indicate that for the period of measurements reported here, and in the absence of major warmings, ozone mixing ratios on an isentropic surface should increase as air parcels cool and bring down the higher mixing ratios from above. As our observations

suggest the opposite of this well-established trend, it appears that, from early January to early February, the ozone variations in the Arctic winter of 1990 were unusual.

Ozone mixing ratio and potential temperature time-altitude contours (isentropes) are also shown in Fig. 1. There is little altitude variation of the isentropes between 15 and 25 km for this period, suggesting the absence of adiabatic vertical motions. Ozone mixing ratios generally decrease along an isentrope in the 24-km (potential temperature of 550 K) region. This reduction in ozone can be explained dynamically by either adiabatic advection of low ozone mixing ratios, which is typically revealed by observable changes in meteorological quantities such as temperature and potential vorticity, or by diabatic advection, under the unusual condition of decreasing ozone mixing ratios with altitude. Typically, diabatic advection results in an ozone increase, because the diabatic circulation brings higher ozone mixing ratios to lower potential temperature values. We will return to these questions later when air-parcel trajectories are discussed.

Figure 2 investigates the ozone mixing ratio variation in more detail, through 1-km averages for the temperature and ozone mixing ratio plotted against time for several altitudes between 18 and 28 km. The first two soundings indicated warm temperatures and low ozone mixing ratios below 22 km, and account for the low total ozone values for these soundings in Fig. 1. As discussed earlier, a general cooling occurred below 24 km during the initial part of the record, but this was interrupted by the 2–3-day warming period on 20 January between 20 and 24 km. Ozone showed a reduction of ~ 1 p.p.m.v. from day 12 to 19 (4 p.p.m.v. per month) between 22 and 26 km, before the 20 January warming. During the warming, it increased to the initial values, but following the return of temperatures below -80°C ,

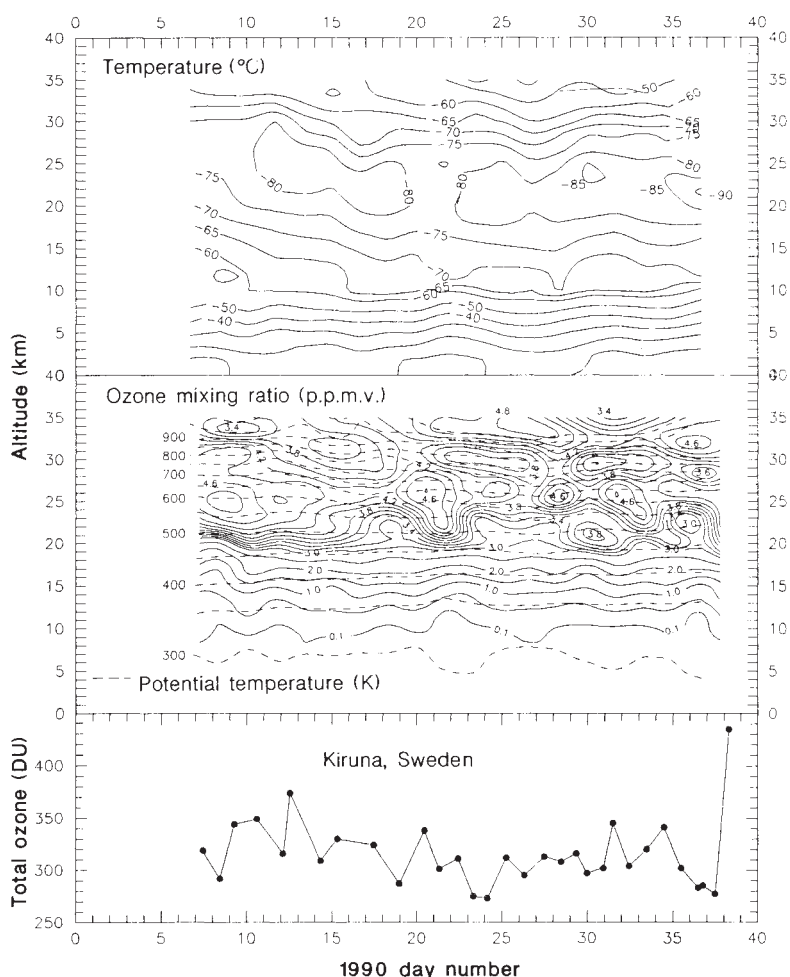
the ozone mixing ratios returned to approximately the same level as before the warming.

An increase in ozone mixing ratio on day 31 is not accompanied by a temperature increase, although another on day 34 is. The final sounding on 8 February, corresponding to rapid motion away from Kiruna of the extremely cold region observed on 6 February, again displays the positive correlation of ozone with temperature, which is probably related to the transport of ozone-rich air from lower latitudes. Grouping the data according to potential temperature, rather than altitude, did not change the general picture in Fig. 2; the initial decline and three ozone maxima on days 21, 31 and 34 remained. This is to be expected because, as indicated in Fig. 1, the potential temperature contours did not display substantial vertical displacement during this time. Neglecting the three peaks in ozone, the remaining data show a gradual reduction of about 0.4 p.p.m.v. between day 18 and 38 (0.6 p.p.m.v. per month) in the 22-km region.

Figure 3 shows the detailed ozone mixing ratio profiles for soundings that reached altitudes above 30 km. The 20–25-km minimum seems to have developed after 13 January, producing a notch in the profile thereafter on every sounding, except those of 22 and 31 January and 3 February, corresponding to the ozone mixing ratio peaks at 22 km on days 21, 31 and 34 in Fig. 2. Even more persistent is an ozone minimum in the 30-km region, which was present to some degree on every sounding that went above this altitude. There is, in addition, the hint of a third minimum in the 35-km region, but insufficient data are available to verify this. These minima, when taken together, suggest a wave in the ozone mixing ratio profile, developing above 20 km, with a wavelength approximately equal to the atmospheric scale height, 6–7 km.

Wind speed and wind direction were measured on some of

FIG. 1 Temperature-altitude contours (top), ozone mixing ratio and potential temperature contours (centre) and total column ozone (bottom) for January and early February 1990 at Kiruna, Sweden. The temperature contour interval is 5°C for temperatures $< -60^\circ\text{C}$ and 10°C for warmer temperatures. The ozone mixing ratio contour interval is 0.2 p.p.m.v. above 3 p.p.m.v. and 0.5 p.p.m.v. below 3 p.p.m.v. The isentrope contour interval is 50 K.



the soundings shown in Fig. 3. On one occasion (24 January) the temperature, wind speed and wind direction showed a clear wave structure with a wavelength of 4–5 km (see Fig. 4). As this is less than the wavelength of the ozone variation, the variations in these meteorological parameters do not seem to be correlated with the ozone variations. It appears that on some occasions, the structure in these meteorological parameters is related to shear layers a few km thick in which the temperature changes by ~ 5 – 10°C , the wind speed changes by $\sim 10\text{ m s}^{-1}$, and the wind direction changes by $\sim 20^\circ$. But data in Fig. 4 suggest that, although such shear layers may result in small ozone perturbations, they are not responsible for the deep minima observed in the ozone mixing ratio at 22 and 30 km. The pronounced, quasi-stationary minimum at 30 km may be related to the presence of the polar jetstream maximum in this region, whereas the minimum at 22 km may not be related to dynamics.

The temperature minima at ~ 19 and 23 km in Fig. 4, although not low enough for water-ice formation, are low enough for NAT condensation for expected values of water and nitric acid vapour concentrations⁸, and PSCs were observed with balloon-borne optical particle counters as indicated by the particle concentration profiles in Fig. 4. The particle measurements indicate that condensation occurred on all available nuclei, resulting in numerous small particles. This was generally the case for the particle measurements at Kiruna in 1990, and appeared to be associated with air-parcel cooling rates of more than 5°C per day. The absence of large NAT particles and the apparently recent production of small particles suggest that the PSC surface area required for the heterogeneous reactions to take place that produce the active chlorine required for ozone depletion, may not have been adequate for substantial depletion to have occurred in these clouds, even in the presence of sunlight. Thus, ozone depletion should not be expected to be precisely

correlated with observed PSC layers. Condensational growth of these small PSC particles in the 18–24-km region, as air parcels cross isotherms during circumnavigation of the pole, could have resulted in the production of a limited amount of active chlorine. A general small reduction in ozone might therefore be expected in air parcels that have spent a long enough time in sunlight.

Air-parcel history

To place these observations in perspective, we have investigated 30-hPa ($\sim 22\text{ km}$) pressure height and temperature maps of the Arctic region produced by the Meteorological Institute of the Free University of Berlin. The daily maps were used to determine the distance from Kiruna of the low-pressure and low-temperature centres of the vortex (the area enclosed by the -80°C and -85°C isotherms), and to estimate geostrophic trajectories in order to determine the latitude and temperature of air parcels before their appearance over Kiruna. The trajectory information was also used to estimate the time an air parcel arriving over Kiruna had spent in sunlight at 20 km (at solar zenith angle $<94.5^\circ$) and within the -80°C isotherm. These results are given in Fig. 5 along with the ozone mixing ratios at 22 km from Fig. 2. Although isentropic trajectory estimates are superior to isobaric, the isentropic surfaces did not vary substantially in the 30-hPa region during the period of measurement (see Fig. 1) and the vortex was relatively uniform throughout the period, thus isobaric trajectories are considered adequate for this analysis.

Although the low-pressure centre was more than $2,000\text{ km}$ from Kiruna for most of the measurement period, the low-temperature centre was generally $<1,500\text{ km}$ from Kiruna, being only several hundred kilometres away on days 18 and 36. After day 12, the cold centre remained relatively stationary with respect to Kiruna, with warmer temperatures upstream. Thus

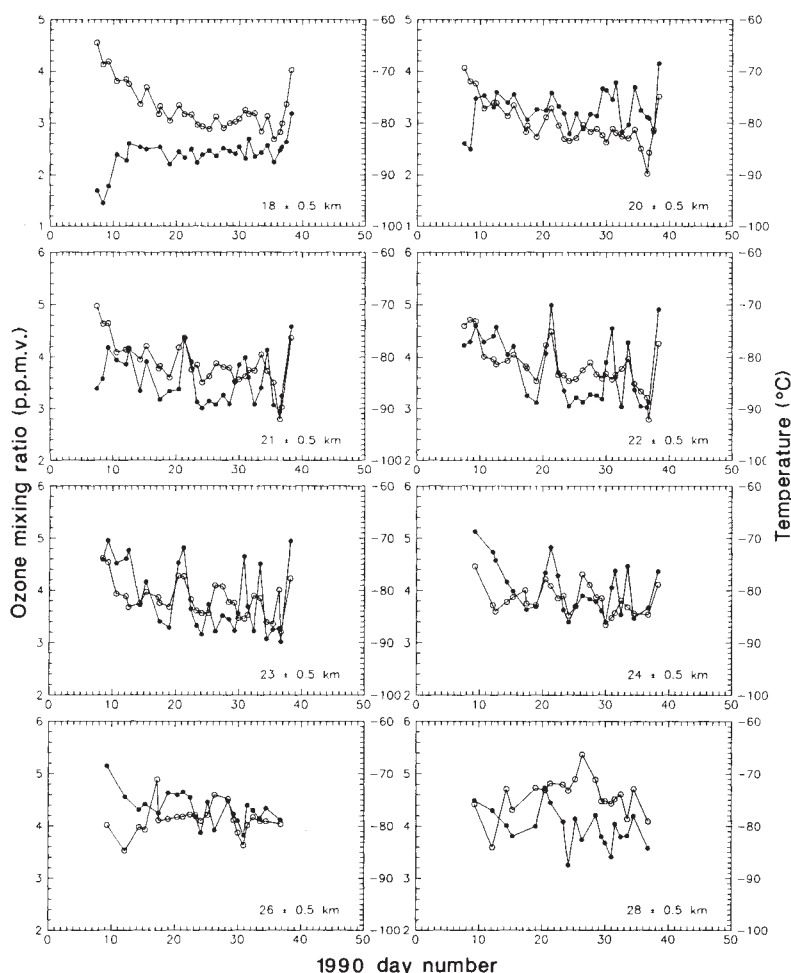


FIG. 2 Temperatures (open circles) and ozone mixing ratios (full circles) averaged over 1 km plotted against time at several altitudes for January and early February 1990 at Kiruna, Sweden.

the temperature reduction observed between days 12 and 24 (see Figs 1 and 2) was not due to the motion of the cold centre towards Kiruna or to cold-air advection, but rather to an intensification of the vortex through diabatic cooling. The gradual increase in the area enclosed by the -80°C , and particularly the -85°C temperature contours, supports this conclusion. Thus, it would appear that quasi-horizontal transport of cold, ozone-poor air cannot explain the observed reductions in ozone. Diabatic cooling typically leads to increases in ozone, as air with higher ozone mixing ratios descends because of the induced subsidence¹². But diabatic cooling may lead to decreases in ozone under the unusual condition of decreasing ozone mixing ratio with altitude (see, for example, the measurements on 6 January 1989³). Ozone-sonde data from Alert, Canada, (82°N , 61.5°W) in January 1990 indicate that the unusual vertical distribution of ozone observed in 1989³ was not repeated in 1990 (data available from the Atmospheric Environment Service, Toronto, Canada). Diabatic cooling should therefore have led to increases in ozone rather than the decreases observed in 1990.

The low ozone values below 22 km on the first two soundings, which resulted in low total ozone for those days, appear to be associated with air from warmer regions at lower latitudes. Between days 12 and 20, when ozone was decreasing between 20 and 24 km, the source region was relatively stationary at a latitude of $\sim 65^{\circ}\text{N}$ and a temperature of $\sim -80^{\circ}\text{C}$, sufficient for ozone depletion, both in terms of exposure to sunlight and presence of PSCs. The ozone maxima at 22 km observed on days 21, 31 and 34 (see Fig. 2) are related to periods when intrusions of warmer air from lower latitudes occurred. The air parcels with the lowest ozone mixing ratios observed in the 22-km region, on days 36 and 37, originated near the North Pole; but at this time the pole was not very cold owing to the shift of the cold vortex towards Scandinavia.

As indicated in Fig. 5, days 37–39 are the only ones for which the air parcel had not spent appreciable time in sunlight during the previous 24 hours. All other trajectories spent between 6 and 9 hours of the previous 24 in sunlight. In most cases, these trajectories passed near the cold dark pole, where chlorine activation by PSCs probably took place, emerged into sunlight for 6 to 9 hours near Greenland and then back into darkness. Also shown in Fig. 5 is the average sunlight per day, at 20 km, for the previous 3–4 days, which is somewhat less than the

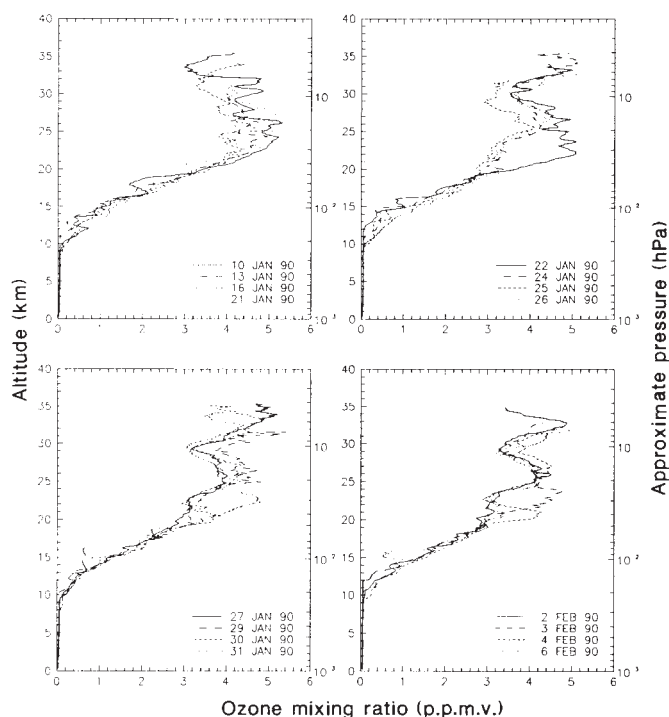


FIG. 3 Ozone mixing ratio profiles for 16 balloon flights between 10 January and 6 February 1990 at Kiruna, Sweden.

previous 24 hours. Perhaps surprising is the fact that this time did not vary much over the measurement period with a value of ~ 3 –5 hours per day. Comparing this with the sunlight during the day before the parcel reached Kiruna shows that until day 37, a large fraction of the solar-exposure time occurred in the 24 hours before sampling. During the circumpolar navigation time of ~ 4 days, the average air parcel spent a total of 12–20 hours in sunlight.

Perhaps the most revealing data in Fig. 5 show the times an air parcel spent at temperatures below -80°C at 30 hPa (~ 22 km) during a polar circumnavigation. Comparing these

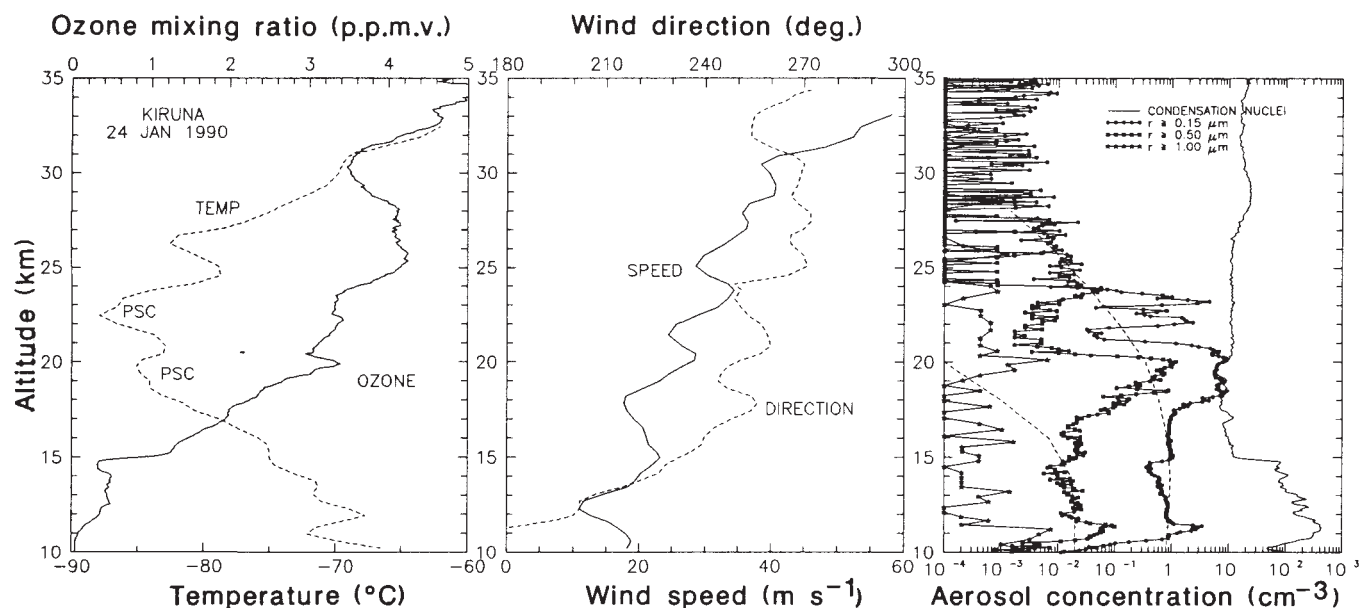


FIG. 4 Temperature, ozone mixing ratio, wind speed and direction, and particle concentration profiles measured on 24 January 1990 at Kiruna, Sweden.

The dashed particle profiles represent the background sulphate profile determined under earlier, warmer conditions⁸.

data with the ozone mixing ratio at 22 ± 0.5 km strongly suggests a negative correlation between the time an air parcel has spent in regions where PSCs could be expected to be present, and ozone mixing ratios. The three ozone mixing ratio peaks at 22 km, days 21, 31 and 34, correspond to minimal times spent by an air parcel at temperatures below -80°C , whereas the minima in the ozone mixing ratio at 22 km correspond to periods when air parcels were at temperatures below -80°C for most of the previous 3–4 days. Because the amount of sunlight on the air parcel during circumnavigation was relatively constant, low temperatures seem to have been the controlling factor in determining the degree of ozone depletion observed in the Arctic in 1990.

In view of the crudeness of the trajectory analysis given here, the relationship of trajectories at 30 hPa and ozone mixing ratio at 22 km is encouraging. More detailed isentropic trajectory calculations could possibly improve the correlation but would probably not add new information. The use of a more detailed computer model for calculating back trajectories might, however, verify the 22-km observation through calculations at other altitudes. For example, as shown in Fig. 2, the variation in ozone mixing ratio between days 9 and 19 at 24 km is somewhat different from that at 22 km, being a more gradual reduction. Although meteorological maps at 24 km (22 hPa) were not available, it is likely that, compared to 22 km, analysis of trajectories in this region would reveal a more gradual increase in time spent at temperatures less than -80°C for days 9–19. A detailed calculation of expected ozone depletion, including

accurate back trajectories as well as chemistry, such as the analysis by McKenna *et al.*¹³ for February 1989, would be a useful extension of this work. The latter analysis estimated a chemical ozone reduction of 20 p.p.b.v. per day along the calculated trajectory under similar conditions in February 1989, a value observed in this work for the non-episodic reduction in late January 1990.

Summary and conclusions

Reductions in ozone that cannot easily be explained by transport processes appear to have occurred in the Arctic stratosphere during the winter of 1990. The conditions required for chemical ozone depletion—PSCs for chlorine activation and sunlight on a portion of the trajectory required for chlorine-catalysed ozone destruction—existed in the 20–26-km region. As in Antarctica in 1987 and 1989⁶, the depletion observed at a single station was episodic with a reduction of ~ 1 p.p.m.v. ($\sim 25\%$) in the 22-km region occurring between days 12 and 19 (4 p.p.m.v. per month, approximately the same rate as the episodic reductions observed in Antarctica). At this time the air-parcel source region was at cold temperatures but relatively low latitudes. This rapid reduction in ozone was followed by a gradual reduction of ~ 0.6 p.p.m.v. per month (about one-third of the non-episodic Antarctic depletion rate). Three superimposed pulses of ozone from warmer low latitudes were observed during this period and finally, on day 38, very warm temperatures and high ozone appeared over Kiruna.

We find that the reductions in ozone seem to be mainly related to the time the air parcel spent at temperatures less than -80°C (the approximate condensation temperature of NAT under existing conditions), suggesting that given some sunlight, the time an air parcel spends in air containing chlorine activated by PSCs was the important parameter in determining the degree of ozone depletion in the Arctic in 1990. Because about half (8 hours) of the total solar exposure during the previous 3–4 days occurred just before our sampling of the air parcel, the actual rate of ozone destruction, if indeed it only took place in sunlight, would be correspondingly larger.

If the episodic reduction in ozone observed in the Arctic is probably of chemical origin and related to the time spent at temperatures less than the condensation temperature of NAT, then a similar conclusion might be drawn for the Antarctic. But because ozone depletion in Antarctica starts at a time when the stratosphere is generally denitrified to some degree, the critical temperature would be less than -80°C . That episodic decreases in ozone were clearly apparent in Antarctica only in 1987 and 1989, when there was a high degree of denitrification, suggests that localized regions of high concentrations of activated chlorine may exist under these conditions only in certain regions where temperatures are low enough to form clouds. This suggests that a more intimate relationship between cloud formation and ozone depletion than generally assumed, may exist under such circumstances. A direct correlation between cloud-particle concentration and ozone depletion was observed in a PSC in Antarctica in September 1988¹⁴.

Being at relatively high altitude in the Arctic, the decreases in ozone mixing ratio reported here do not dramatically affect total ozone. The reduction of $\sim 10\%$ in total ozone between days 12 and 19 is largely caused by the reduction in mixing ratio in the 22–24-km region, which appears not to be related to transport, but to the time the relevant air parcel has spent at temperatures less than -80°C . It is likely that chemical ozone depletion in the Arctic spring occurs in a doughnut- or crescent-shaped region determined by trajectories of air parcels that have spent enough time at temperatures below -80°C for chlorine activation to take place, and have subsequently been exposed to sunlight. Occasional injections of ozone from lower latitudes would appear as an interruption in chemical ozone depletion so that although the observed reduction may not seem severe, the total amount of ozone being destroyed may be substantial.

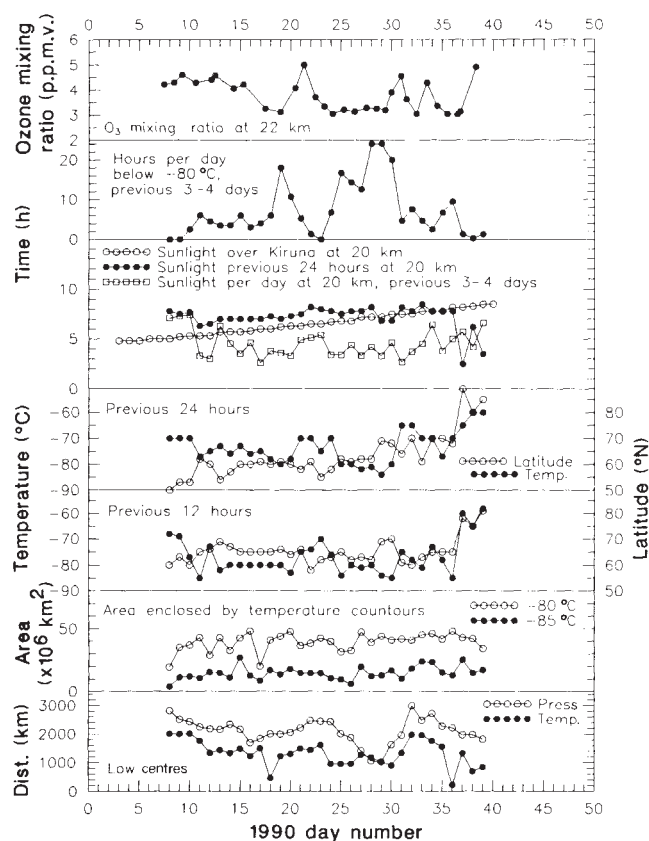


FIG. 5 The panels show, for the 30-hPa level, the distance of the polar low-pressure and temperature centres from Kiruna; the area enclosed by the -80°C and -85°C isotherms; the latitude and temperature of air parcels 12 and 24 hours before reaching Kiruna; the sunlight hours at 20 km over Kiruna, the sunlight hours for an air parcel at 20 km during the 24 hours before reaching Kiruna, average sunlight per day on an air parcel at 20 km for the 3–4 days before reaching Kiruna; average time per day an air parcel has spent at temperatures less than -80°C during the 3–4 days before reaching Kiruna, and the ozone mixing ratio at 22 ± 0.5 km.

Unless the flow is highly non-zonal so that air parcels that have been exposed to sunlight appear at very high latitudes, the ideal place to study Arctic ozone depletion may be at the periphery, rather than inside the cold dark vortex. Because the

intensification of the Aleutian high-pressure system in January results in a general movement of the vortex to the European side, northern Scandinavia appears to be ideally situated for such studies. □

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Alteration of voltage-dependence of *Shaker* potassium channel by mutations in the S4 sequence

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Voltage-dependent potassium, sodium and calcium ion channels may share a common mechanism of activation, in which the conserved S4 sequence acts as the primary voltage sensor. Site-directed mutagenesis of the S4 sequence of the *Shaker* potassium channel and electrophysiological analysis suggest that voltage-dependent activation involves the S4 sequence but is not solely due to electrostatic interactions.

VOLTAGE-activated ion channels control the permeability of cells to specific ions by opening or closing in response to changes in the potential difference across the plasma membrane¹. Channel opening is thought to be controlled by voltage sensors, charged or polar structures intrinsic to the channel protein which detect a change in membrane potential and respond to it by moving in the electric field, thereby causing conformational changes that lead to channel opening. The equivalent of about four to six charges (gating charges) are translocated across the membrane field during the activation of Na⁺, Ca²⁺ and K⁺ channels^{1,2}. Indeed, movements of charges intrinsic to the channel molecules (gating currents) have been detected during the activation of Na⁺ and K⁺ channels^{3,4}.

Voltage-dependent Na⁺, Ca²⁺ and K⁺ channels contain a common motif, the S4 sequence^{5–8}, a structure with basic amino acids (arginine or lysine) at every third or fourth position. The S4 sequence has been proposed to function as a voltage sensor^{6,9–12}. In this hypothesis, the positively charged amino acids of the S4 sequence are located within the membrane electric field, where they may be stabilized by forming ion pairs with acidic amino acids elsewhere in the channel protein. Depolarization of the membrane propels the positively charged

S4 sequence towards the outside of the cell, causing a conformational change that opens the channel. According to this hypothesis, the S4 charges (and perhaps their ion-pair partners) contribute to the channel's gating charge and their movement constitutes the gating current. Mutations of basic residues in the S4 sequence of the Na⁺ channel have been reported to alter its gating charge¹³. A model similar to the S4 hypothesis was proposed before the sequences of these channels were known¹⁴.

To test the S4 hypothesis, we have made site-directed mutations of the voltage-dependent K⁺ channel encoded by the *Shaker* gene of the fruit fly *Drosophila melanogaster*^{5,15–17}. This gene produces, by alternative splicing, several proteins which form functional K⁺ channels after expression in *Xenopus* oocytes^{18–23}. *Shaker* K⁺ channels open with depolarization, but then enter a refractory state (inactivate) if the depolarization is maintained. *Shaker* proteins contain one S4 sequence (Fig. 1a)^{5,18–20}. In the Na⁺ and Ca²⁺ channels, which resemble four *Shaker*-like proteins hooked together, there are four S4 sequences^{6–8}. Therefore, the *Shaker* K⁺ channels formed in oocytes are thought to be multimers composed of several subunits^{24–26}.

We have made mutations of each of the seven basic amino acids in the S4 sequence of the *Shaker* cDNA clone ShB (refs. 15, 18), expressed the mutant channels in *Xenopus* oocytes, and analysed the currents using a two-electrode voltage clamp. The mutations were of two kinds. First, each basic amino acid in the S4 sequence was changed individually to the neutral amino acid glutamine. If the S4 amino acids constitute the channel's entire gating charge, and if they contribute equally to it, then removing one of the seven positively charged S4 amino acids per subunit would reduce the sensitivity of the channel to voltage by ~15%. If other amino acids (such as acidic amino acids ion-paired to the S4 basic residues) also contribute to the gating charge, the effect would be less. In the second kind of mutation, arginine residues were replaced one at a time by lysine, or lysine by arginine. These mutations involve no change in charge on the S4 sequence, and are not expected to affect the channel's sensitivity to voltage.

We tested the proposal that the S4 basic amino acids of the *Shaker* K⁺ channel constitute part or all of the gating charge

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and that they contribute equally to it using three methods to measure the voltage-dependence of activation. Our results indicate that the S4 sequence is important for voltage-dependent gating. We find, however, that the effects of the substitutions were not equivalent at the seven positions, and that the sensitivity of the channel to voltage can be reduced without removing charges from the S4 sequence. Activation of the K⁺ channel cannot be explained completely by electrostatic interactions between the S4 basic amino acids and the membrane electric field.

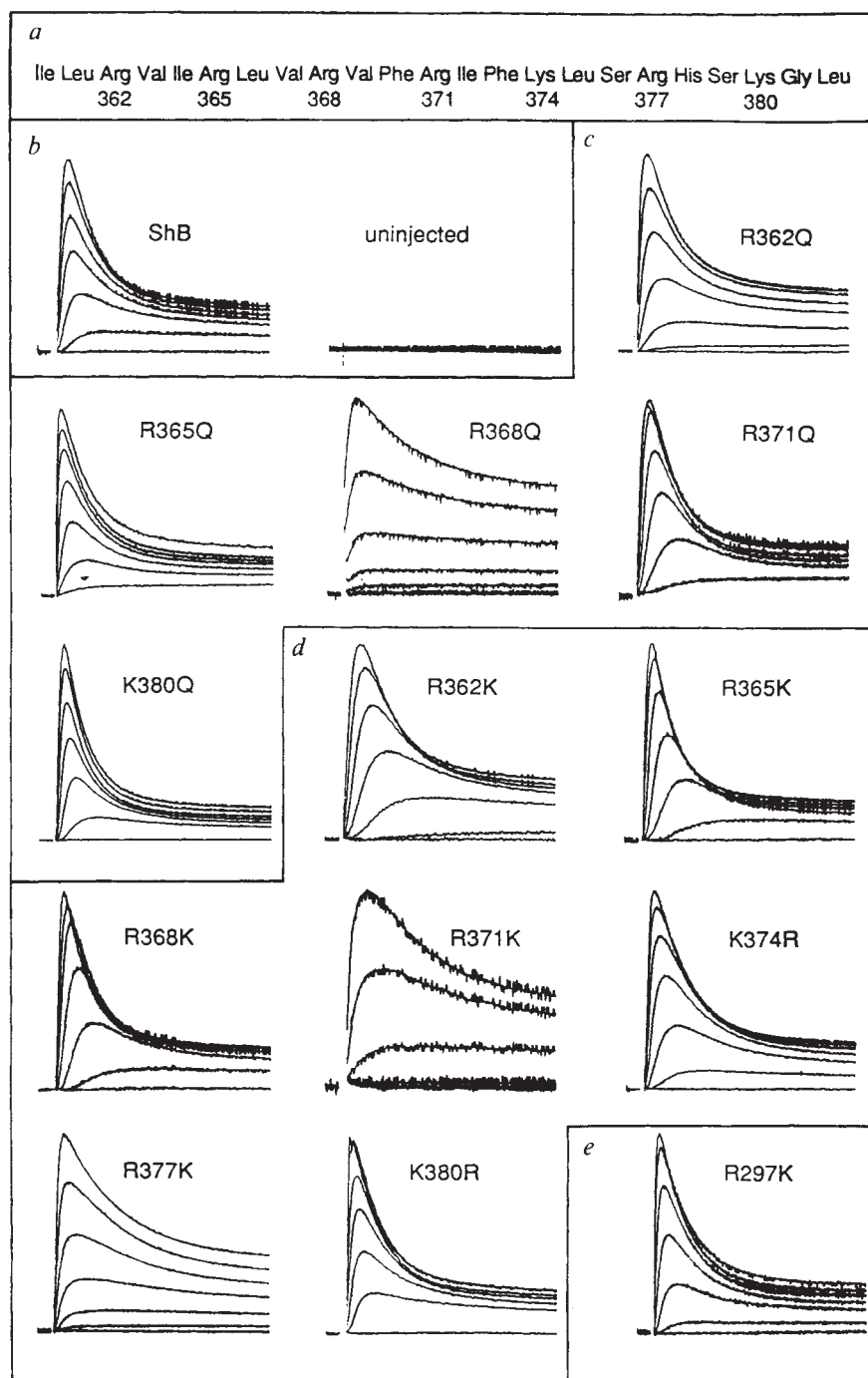
FIG. 1 Transient outward currents of ShB and mutant channels in oocytes. *a*, S4 sequence of the *Shaker* K⁺ channel (for complete sequence, see ref. 18). The basic amino acids of the S4 sequence were replaced one at a time by Gln, or Arg was replaced by Lys or Lys by Arg. Outward currents, recorded at 12°C using a two-electrode voltage clamp^{21,22}, were evoked by steps from -80 mV to potentials between -40 and +80 mV in 20 mV increments. The currents at +80 mV have been scaled to the same amplitude; their actual peak current amplitudes at +80 mV ranged from 3 to 15 µA. A linear leak current has been subtracted. Each current trace is 85 ms. Currents are shown from oocytes expressing: *b*, ShB or no *Shaker* channels (uninjected); *c*, neutralization mutations R362Q, R365Q, R368Q, R371Q and K380Q. Two others, K374Q and R377Q, did not produce functional channels (not shown). *d*, Basic substitution mutations R362K, R365K, R368K, R371K, K374R, R377K and K380R. *e*, A mutation R297K in hydrophobic segment H2 (ref. 18). R297Q did not produce functional channels (not shown).

METHODS. The ShB1 cDNA¹⁸ was subcloned into the plasmid expression vectors SP72 (Promega) or Bluescript (Stratagene). Single amino-acid mutations were made by two methods. Cassette mutagenesis³⁵ was used to make mutations at positions K374 and R377 in the SP72 subclone. The subclone was cut by *Bst*B1 at nucleotides 1,110 and 1,135 (see ref. 18 for nucleotide numbering). Complementary oligonucleotides containing the desired mutations and overhanging *Bst*B1 ends were synthesized, phosphorylated with T4 polynucleotide kinase, annealed, and ligated into ShB1 in place of the original sequence by standard procedures³⁶. Oligonucleotide-directed mutagenesis of a uracil-substituted single stranded template³⁷ was used to make mutations in the Bluescript subclone. Template DNA was prepared from the *dut*⁻*ung*⁻ strain RZ1032. A phosphorylated mutagenic oligonucleotide 24–26 bases in length was annealed to the template DNA at an approximate molar ratio of 10:1 (ref. 37) and was extended by Sequenase or T4 DNA polymerase and single-stranded binding protein (US Biochemicals). After transformation into HB101, mutant clones were identified by colony screening using the mutagenic oligonucleotide as probe, and subsequently by double-stranded DNA sequencing^{38,39}. The following controls indicated that mutations at sites other than the intended sites were unlikely. Each mutagenic oligonucleotide, annealed to the template DNA as described above, was used as a sequencing primer, resulting in clearly readable sequence with no detectable second site priming (data not shown). All the mutant clones used here were sequenced in a region of 200–400 bases around the site of the primary mutation and did not contain additional mutations. Two or more independent clones were isolated for each channel mutant and were found to have the same electrophysiological phenotype. The last two controls also apply to the mutations derived by double-stranded cassette mutagenesis. RNA was transcribed and capped *in vitro* from ShB or mutant subclones as previously described^{21,22}, except that RNA from the Bluescript subclone was transcribed

Voltage-dependence of mutated channels

Five of the seven glutamine substitution mutations produced functional K⁺ channels which conducted transient outward currents (Fig. 1*b, c*). Two mutations, K374Q and R377Q, did not produce functional channels, and were not studied further.

Voltage-dependence of activation was characterized by measuring peak conductance as a function of voltage (Fig. 2*a*, top). The slope of this conductance-voltage (*g*-*V*) curve provides an estimate of gating charge¹. To characterize the voltage-dependence of inactivation of the whole-cell current, we applied



by T3 RNA polymerase. Sufficient mutant or ShB RNA to give 1–15 µA peak currents at +80 mV was injected into oocytes²¹. Voltage-clamp experiments were performed as previously described^{21,22} except that 75–90% of the measured series resistance (500 Ω) was compensated. Oocytes were bathed in modified Barth's saline²¹, containing 1 mM K⁺, unless otherwise noted.

TABLE 1 Properties of ShB and mutant channels in *Xenopus* oocytes.

	V_{mid} (mV)*	Activation Slope (mV)*	Limiting slope (mV)†	V_{mid} (mV)‡	Inactivation Slope (mV)‡	τ_{h1} (ms) onset§	τ (ms) recovery at -80 mV	Ion selectivity V_r (mV) in 1 mM K ⁺ ¶	V_r (mV) in 40 mM K ⁺ ¶
ShB	-10 ± 4 (7)	14 ± 1 (7)	6.0 ± 0.7 (7)	-29 ± 2 (4)	4.9 ± 1.2 (4)	9.8 ± 1.1 (8)	49 ± 14 (7)	-76 ± 9 (7)	-23 ± 9 (5)
Neutralization									
R362Q	9 ± 6 (4)	15 ± 1 (4)	5.1 ± 2.3 (4)	-7 ± 1 (4)	9.1 ± 0.3 (4)	12.4 ± 0.3 (3)	46 ± 1 (4)	-70 ± 4 (6)	-23 ± 2 (3)
R365Q	-21 ± 8 (7)	15 ± 4 (7)	7.1 ± 2.6 (3)	-43 ± 4 (3)	7.8 ± 1.7 (3)	8.2 ± 1.3 (4)	44 ± 2 (5)	-71 ± 5 (6)	-22 ± 4 (5)
R368Q	41 ± 12 (7)	20 ± 6 (7)	49 ± 18 (4)	26 ± 5 (5)	17.0 ± 1.9 (6)	22.2 ± 4.4 (6)	44 ± 6 (5)	-58 ± 9 (6)	-21 ± 5 (3)
R371Q	-27 ± 10 (5)	17 ± 5 (5)	3.7 ± 0.8 (2)	-43 ± 3 (5)	5.3 ± 2.3 (5)	13.6 ± 0.7 (5)	62 ± 11 (4)	-81 ± 12 (7)	-23 ± 6 (3)
K380Q	-5 ± 2 (8)	15 ± 2 (8)	8.0 ± 3.6 (3)	-33 ± 2 (5)	4.5 ± 0.4 (5)	9.2 ± 0.8 (5)	53 ± 5 (3)	-70 ± 7 (7)	-23 (1)
Basic substitution									
R362K	1 ± 4 (4)	12 ± 2 (4)	4.1 ± 1.9 (6)	-17 ± 4 (4)	4.1 ± 0.7 (4)	12.1 ± 1.0 (5)	44 ± 3 (6)	-61 ± 6 (11)	-21 ± 5 (5)
R365K	-3 ± 6 (6)	16 ± 1 (6)	4.3 ± 0.5 (4)	-25 ± 7 (4)	5.1 ± 0.3 (4)	12.0 ± 2.8 (4)	52 ± 11 (4)	-69 ± 7 (7)	-21 ± 2 (3)
R368K	-3 ± 5 (8)	16 ± 4 (8)	5.0 ± 1.7 (8)	-37 ± 2 (5)	4.6 ± 1.2 (5)	9.4 ± 0.2 (3)	129 ± 28 (4)	-88 ± 9 (8)	-28 ± 2 (4)
R371K	52 ± 5 (13)	10 ± 2 (13)	5.8 ± 1.3 (2)	35 ± 5 (6)	8.8 ± 1.4 (6)	19.3 ± 4.3 (5)	45 ± 2 (4)	ND	ND
K374R	-8 ± 4 (9)	13 ± 2 (9)	5.2 ± 1.6 (8)	-31 ± 1 (3)	6.4 ± 0.8 (3)	9.7 ± 1.8 (4)	55 ± 4 (6)	-70 ± 10 (9)	-21 ± 3 (3)
R377K	21 ± 5 (10)	18 ± 3 (11)	6.2 ± 2.7 (5)	-2 ± 7 (4)	15.0 ± 1.6 (4)	16.7 ± 5.7 (6)	40 ± 6 (4)	-59 ± 6 (8)	-23 ± 6 (4)
K380R	-16 ± 5 (9)	11 ± 2 (9)	6.1 ± 1.3 (5)	-32 ± 2 (6)	4.2 ± 0.8 (6)	10.7 ± 2.2 (5)	46 ± 3 (4)	-73 ± 9 (15)	-22 ± 2 (3)
Outside S4									
R297K	-2 ± 5 (7)	13 ± 1 (7)	5.9 ± 0.8 (3)	-15 ± 4 (6)	8.4 ± 1.7 (6)	12.3 ± 2.4 (3)	43 ± 13 (5)	-60 ± 5 (7)	-19 ± 3 (4)

* Conductance-voltage relationships were fit to Boltzmann distributions, as described in Fig. 2, to derive values for the midpoint (V_{mid} , mV) and the slope (mV/ e -fold change in g/g_{max}) of the curves.

† To derive the limiting voltage-sensitivity (mV/ e -fold change in g/g_{max}), the logarithm of the normalized conductance was plotted against voltage. A line was fit by eye to conductance values below 10% of g_{max} , and the slope of the line was determined. This measurement was difficult to make for R365Q and R371Q, which activated close to the reversal potential of the current.

‡ Prepulse inactivation-voltage relationships were fit to Boltzmann distributions to derive values for the V_{mid} (mV) and the slope (mV/ e -fold change in $I_p/I_{p,max}$) of the curves.

§ The main time constant for the onset of inactivation, τ_{h1} (ms), was determined at +80 mV, except for R365Q, measured at +40 mV, (Fig. 4); τ_{h1} accounted for 65–94% of the inactivation of ShB and mutant channels. For mutants shifted far in the depolarized direction, τ_{h1} had not reached a minimum value at +80 mV (Fig. 4).

|| Recovery from inactivation at -80 mV was measured using a two-pulse protocol as previously described²². The conditioning and test pulses were to +20 mV, except in highly shifted mutants where +60 mV was used. Recovery data were fit with two time constants; the main component is reported here.

¶ Reversal potential of the current, V_r (mV), was determined in 1 or 40 mM external K⁺ as previously described²¹ except that tail currents were evoked every 5 mV in the region of reversal potential, which was assigned by eye to the nearest 5 mV. V_r in 1 mM K⁺ varies from ~-55 to -85 mV between batches of oocytes, independent of which mutant was expressed. This effect was presumably due to variation of the internal K⁺ concentration. V_r was not determined for R371K. Inward currents or tail currents were not observed in 1, 40 or 115 mM K⁺ in this mutant, which did not begin to activate until ~+20 mV. In R371K, tail currents from -80 to 0 mV were too fast to resolve.

All values are given as mean ± s.d. (ND, not determined). Figures in brackets, numbers of independent determinations. The three methods we have used to quantify the sensitivity of the channel to voltage, slope of g - V , prepulse inactivation and limiting voltage sensitivity, may not be equally reliable as they depend on different assumptions. To estimate the channel's gating charge from the slope of the g - V curve, we assume that the voltage sensor is in equilibrium at the peak current. This may not be valid because the channel inactivates. Although estimates of gating charge from the slope of the g - V curve may be subject to error because of inactivation, changes in the slope of the g - V curve in mutant channels are not likely to result from changes in the kinetics of inactivation, which are not significantly affected by the mutations. The assumption implicit in the use of the Boltzmann distribution to fit the g - V and prepulse inactivation data is that two states suffice to describe activation (closed and open) or inactivation (open and inactivated)⁴. Theoretically, the limiting voltage-sensitivity provides a better estimate of the channel's gating charge, because it does not require any assumptions about the number of closed states in the activation process²⁷. Our values for the limiting voltage-sensitivity are derived from currents which represent between 1 and 10% of the maximal conductance of the channel. More accurate estimates of gating charge may require the ability to detect smaller currents, which is not technically feasible. Therefore, our measurements probably underestimate the gating charge of the channel. The slope of the prepulse inactivation curve may be the most reliable estimate²; it predicts a slightly higher gating charge for ShB channels than does the limiting voltage-sensitivity, and is independent of the linearity of the open-channel current-voltage relationship.

depolarizing prepulses to different potentials and then determined, with a subsequent test pulse, the fraction of channels inactivated by the prepulse (Fig. 2a, bottom). To quantify the midpoints and slopes of the g - V and prepulse inactivation curves, the data were fit to Boltzmann distributions (Fig. 2a, Table 1)¹. The g - V data tended to deviate from the Boltzmann fits at small depolarizations, so we also determined the limiting voltage-sensitivity (the slope of the g - V curve at its steepest point, just above threshold for activation) to provide another estimate of gating charge (Table 1)²⁷.

Four neutralization mutations did not significantly alter the slope of the g - V curve or the limiting voltage-sensitivity, although several shifted the midpoint of the g - V curve in the depolarized (R362Q) or hyperpolarized (R365Q and R371Q) direction. By contrast, the mutation R368Q decreased the slope of the g - V curve by 40% and shifted the midpoint of activation in the depolarized direction. The limiting voltage-sensitivity was decreased eightfold (Table 1). This reduction in voltage-dependence in R368Q is much larger than the effect predicted by the S4 hypothesis.

All seven basic substitution mutations produced functional channels (Fig. 1d). Four (R365K, R368K, K374R and K380R) did not significantly alter the g - V curve, but two shifted the midpoint of activation in the depolarized direction, without detectably changing the slope of the g - V curve. R362K caused a small shift, whereas R371K shifted the midpoint of activation by ~60 mV (Fig. 2b, Table 1).

R377K had an unexpected effect: This mutant was substan-

tially less sensitive to changes in voltage than its parent ShB, decreasing the slope of the g - V curve by 30%, an amount nearly as large as that seen in R368Q (Fig. 2b). However, unlike R368Q, the limiting voltage-sensitivity of the channel was not altered (Table 1).

Coupling of activation and inactivation

Mutations which shift the midpoint of activation shift the midpoint of prepulse inactivation by about the same amount (Fig. 2; Fig. 3). Furthermore, R368Q and R377K, which decrease the slope of the g - V curve, also decrease the slope of the prepulse inactivation curve by 250 and 200%, respectively (Fig. 2; Table 1). These results confirm previous reports that activation and inactivation are strongly coupled in the *Shaker* K⁺ channel: that is, most channels open before they inactivate^{2,28}.

Mutations that shift activation and inactivation also shift the voltage-dependence of τ_{h1} , the time constant of the main exponential component of the decay phase of the current, by about the same amount (Fig. 4). In ShB channels²¹, and in most of the mutants, τ_{h1} decreased with increasing depolarization until it reached a minimum, voltage-independent value related to the single-channel inactivation rate (see Fig. 4). Many mutant channels reached minimum values for τ_{h1} similar to that attained by ShB (Table 1), indicating that the single-channel inactivation rate is not dramatically changed.

For those mutants with the largest shifts in voltage-dependence, we were unable to measure a minimum value for τ_{h1} (Fig. 4). But the shifts in τ_{h1} against voltage curves were

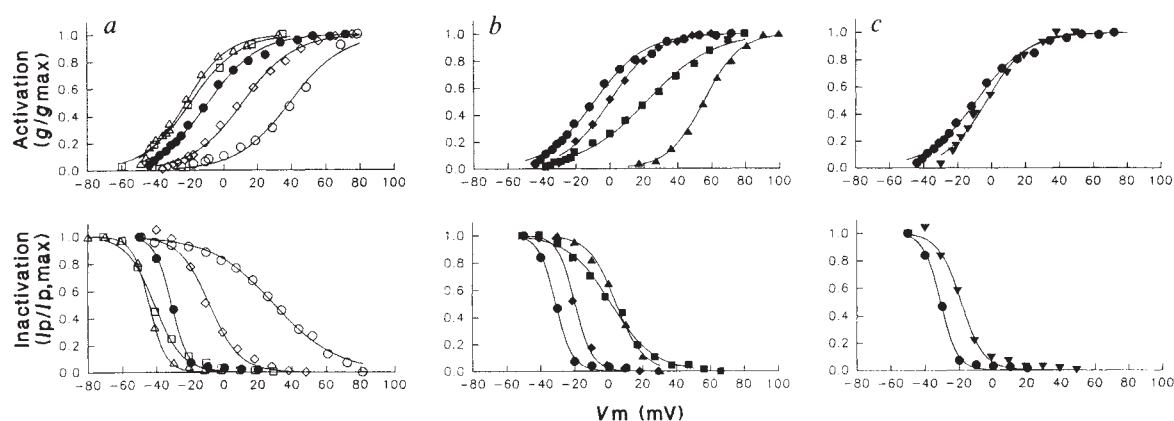


FIG. 2 Voltage-dependence of activation and prepulse inactivation of ShB and mutant channels. Top, conductance-voltage curves, and bottom, prepulse inactivation-voltage curves for ShB (●) and mutants which affected the voltage-dependence of the channel. *a*, Open symbols, neutralization mutations: R362Q (◇), R365Q (□), R368Q (○) and R371Q (△); *b*, filled symbols, basic substitution mutations: R362K (◆), R371K (▲) and R377K (■); *c*, R297K (▼), in hydrophobic segment H2 (ref. 18). Symbols, data from single representative experiments. Solid curves, best-fit Boltzmann distributions. METHODS. Single-channel current-voltage relation of *Shaker* channels in cultured fly myocytes is approximately linear between -20 mV and $+80$ mV (ref. 40). For g - V curves, the amplitude of the leak-subtracted peak current was determined as a function of voltage and divided by the driving force¹; the reversal potential of the current, V_r , was determined for each oocyte (Table 1), where feasible. V_r could not be determined for R371K (Table 1), so a value of -65 mV was assumed. Conductance values were normalized to the maximal conductance measured in that oocyte and plotted against voltage. Midpoints and slopes were determined by a nonlinear least-squares fitting procedure to a Boltzmann distribution. A two-state Boltzmann distribution was chosen because it provided a good fit and because it involved the fewest *a priori* assumptions about the gating mechanisms. For prepulse inactivation, a 500 ms prepulse at the indicated potential was followed by an 80 ms test pulse to $+80$ mV (or $+100$ mV for some mutants which

shifted the curve in the depolarized direction). The peak current during the test pulse was normalized to the maximal peak current determined after a prepulse to -50 mV, or -80 mV for mutants which shifted the curve in the hyperpolarized direction. A noninactivating, steady-state component of the current which remained at the end of the most depolarized prepulse has been subtracted. The adjusted, normalized current was plotted against prepulse potential, and the data fitted to a Boltzmann distribution. For the experiments shown, the noninactivating component as a per cent of the maximal peak current was: ShB (22), R362Q (21), R365Q (4), R368Q (29), R371Q (29), R362K (19), R371K (30), R377K (27), R297K (18). The holding potential in all experiments was -80 mV. Several technical limitations may have affected our ability to characterize quantitatively the mutants shifted the furthest in the depolarized or hyperpolarized directions. First, R365Q and R371Q, which are shifted in the hyperpolarized direction, activated close to the reversal potential of the current in 1 mM K^+ . Second, the conductance of the ShB and mutant channels often declined at large depolarizations (not shown). The decline, which may have interfered with determining the maximal conductance of the most depolarized mutants in some oocytes, may have been due to internal Na^+ attempting to leave the cell through the K^+ channel, blocking it in the process. Third, the mutant channels shifted furthest in the depolarized direction did not always reach their maximal conductances by $+100$ mV, the largest depolarization we used.

consistent with the idea that apparent changes in the kinetics of macroscopic inactivation (see Fig. 1) result primarily from shifts in the voltage-dependence of the channel. Although mutations which shift activation in the depolarized direction slow inactivation relative to ShB when compared at the same membrane potential, the kinetics of inactivation are very similar when compared at equivalent levels of activation.

Previous single-channel analyses have shown that most of the voltage-dependence of the *Shaker* K^+ channel resides in the activation process, and that inactivation is voltage-independent over a large range of potentials^{2,28}. Therefore, the voltage sensor of the *Shaker* channel is likely to control primarily activation. The apparent voltage-dependence of prepulse inactivation reflects the underlying voltage-dependence of activation. In this case, the slope of the prepulse inactivation curve provides another estimate of the activation gating charge (Table 1).

Specificity of S4 mutations

Our results indicate that the S4 mutations alter the voltage-dependent properties of the channel. To determine whether mutations have general effects on channel function due to gross effects on channel structure, we examined the recovery from inactivation and the K^+ selectivity. The rate of recovery from inactivation at -80 mV was normal in 11 out of 12 of the functional S4 mutant channels (Table 1). In one, R368K, the rate was almost three times slower than in ShB, although it was still much faster than in the *Shaker* alternative splice variants, ShA and ShC, which require minutes at -80 mV to recover from inactivation^{21,22}. The selectivity for K^+ was determined by measuring the reversal potential of the current in two different external K^+ concentrations, 1 and 40 mM. The reversal potential

of the most highly shifted mutant, R371K, could not be determined (Table 1). The other functional S4 mutations had little effect on the reversal potential (Table 1), indicating that S4 mutations specifically alter the voltage-dependence of the K^+ channel and not the global channel structure.

Are the S4 basic amino acids the only residues in the channel that affect its voltage-dependence? There is one other basic amino acid, R297, predicted by hydropathy analysis to be in a membrane-spanning segment (hydrophobic segment H2)^{5,18}. The mutation R297Q did not produce functional channels, whereas R297K caused a small depolarizing shift of the activa-

TABLE 2 Summary of mutant phenotypes

Position	Neutralization	Basic substitution
R362	+20 mV	+12 mV
R365	-12	Like ShB
R368	+53, less sensitive to V	Like ShB
R371	-16	+64
K374	No function	Like ShB
R377	No function	+29, less sensitive to V
K380	Like ShB	Like ShB
R297 (in H2)	No function	+11

Values (in mV) indicate the average shift of the midpoints of activation and inactivation in mutant channels, relative to ShB channels. Positive values, shifts in the depolarized direction, negative values, shifts in the hyperpolarized direction. Left column, neutralization mutations have Gln instead of the original basic amino acid. Right column, basic substitution mutations have Lys instead of the original Arg, and vice versa. Effects on the voltage-dependence of the channel are noted.

tion and inactivation curves without altering their slopes (Figs 1e, 2c). Like the S4 mutants, R297K did not affect other functional properties of the channel (Table 1). Thus, the S4 residues are not the only amino acids in the channel that specifically affect its voltage-dependence.

Test of the S4 hypothesis

Mutations in the S4 sequence specifically shifted the midpoints of activation and inactivation and decreased the channel's gating charge as estimated from the slopes of the $g-V$ and prepulse inactivation curves and the limiting voltage-sensitivity. Qualitatively, these results are consistent with the idea that the S4 basic amino acids are part of the voltage sensor of the channel. Quantitatively, however, our results differ from predictions of the S4 hypothesis in two ways. First, neutralizations of basic residues at four S4 positions did not detectably reduce the estimated gating charge. By contrast, neutralization of R368 reduced the channel's sensitivity to voltage much more than expected if the seven S4 basic amino acids contribute equally to the gating charge. Second, the mutation R377K also dramatically reduced the channel's sensitivity to voltage by two criteria, even though this substitution should not reduce the charge on the S4 sequence.

Our three methods to quantify the sensitivity of the channel to voltage may not be equally reliable as they rely on different assumptions (see Table 1). Further study, including single-channel analysis and gating-current measurements, is required to understand why the different estimates of gating charge were differentially affected by the mutations. Our results indicate, however, that activation of the channel cannot be explained by simple S4 models, including models in which the basic residues contribute identically to the total gating charge.

The effects of R368Q and R377K on the voltage-dependence of the channel were not proportional to the charge on the S4 sequence, perhaps because these mutations restrict the movement of the voltage sensor through the membrane electric field. If the charges on the voltage sensor do not move as far through the membrane field in these two mutants, the channel will be less voltage-dependent by a factor larger than that due to the actual change in charge generated by the mutation. However, partial movement of the S4 sequence would have to be sufficient to trigger the conformational change that opens the pore.

Another explanation is that cooperative interactions contribute to channel activation. In ShB channels, the voltage-dependent movement of an S4 sequence in one subunit may increase the likelihood that the S4 segment in another subunit makes the same transition. In this case, the steepness of the

activation curve of ShB would be due partly to the charge on the voltage sensor and partly to interactions between subunits. If the mutations reduced the cooperativity between subunits, the slope of the $g-V$ curve would be reduced to reflect the voltage-sensitivity of the gating structure in the absence of cooperativity.

Shifts in activation and inactivation

The most frequently observed effect of the S4 mutations (5 out of 14) and the effect of a mutation outside the S4 sequence, R297K, was to shift the midpoints of activation and inactivation without detectably changing the slopes of the $g-V$ and prepulse inactivation curves (Table 2). One likely explanation is that these mutations change the stability of the open or closed states of the channel, or both, altering the equilibrium distribution between them and thereby shifting the channel's voltage-dependence. Mutations of the voltage sensor could have this effect, but so could mutations of other amino acids important in stabilizing the open and closed conformations. Therefore, shifts of activation and inactivation midpoints are consistent with the idea that the S4 amino acids are part of the channel's voltage sensor, but do not exclude other possibilities.

The authors of a previous report, citing an inverse relationship between gating charge and the number of charges removed from S4, concluded that the S4 sequence is part of the voltage sensor in Na^+ channels¹³. Our conclusions differ as follows. First, we did not observe a consistent correlation between the number of S4 charges neutralized and changes in the estimated gating charge. Similar discrepancies may occur in the Na^+ channel where neutralizing one of 24 S4 basic residues reduced the gating charge by amounts ranging from zero to 25% (ref. 13). Second, Na^+ -channel mutations shifted activation and inactivation along the voltage axis, but apparently not in parallel¹³, despite the fact that biophysical studies have demonstrated strong coupling between activation and inactivation in Na^+ channels²⁹⁻³⁴. Third, because neutralization of basic amino acids at the carboxyl end of an S4 sequence of the Na^+ channel shifted activation in the depolarized direction, whereas neutralizations at the amino end of the same S4 sequence shifted activation in the hyperpolarized direction, it was proposed that the mutations caused the shifts by altering the electric field detected by the S4 voltage sensor¹³. This idea does not explain our results, because neutralization of the first four basic residues in the S4 sequence of the *Shaker* K^+ channel alternated in shifting the voltage dependence of activation in the depolarized and hyperpolarized directions and because basic substitutions caused similar shifts (Table 2).

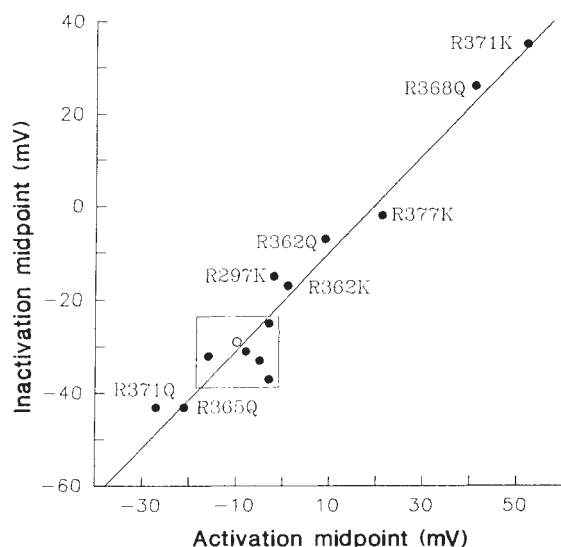
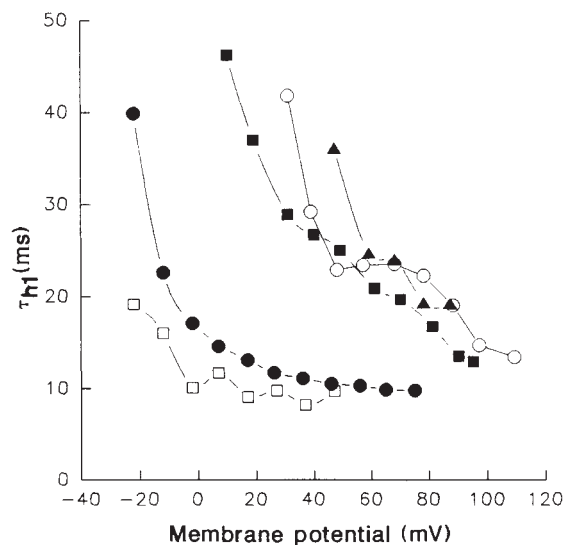


FIG. 3 Activation and inactivation are strongly coupled in the *Shaker* K^+ channel. Midpoints of the $g-V$ curves of ShB and mutant channels have been plotted against the midpoints of prepulse inactivation curves. Each value represents the average of three or more determinations (see Table 1 for s.d. and N). The line, fit by linear regression (correlation coefficient 0.97), has a slope of 1.03. Box, midpoints of ShB (open circle) and those mutations which did not significantly alter the channel's voltage-dependence: R365K, R368K, K374R, K380R and K380Q (closed circles).

FIG. 4 Mutations that shift the voltage-dependence of activation also shift the voltage-dependence of the rate of macroscopic inactivation. The fast time-constant of the decay phase of the current, τ_{h1} (ref. 22), has been plotted as a function of voltage for ShB (●) and four shifted mutant channels, R365Q (□), R368Q (○), R371K (▲), and R377K (■). Each point represents the average of determinations from 3–6 experiments. At large depolarizations, the activation rate should be very fast, contributing little to τ_{h1} . Therefore, as a first approximation, the minimum, voltage-independent value for τ_{h1} should be determined by the rate constants between the open state, O , and an inactivated state, I , which is nonabsorbing since inactivation in the ShB and mutant channels is incomplete. For $O \xrightleftharpoons[a]{b} I$, $\tau_{h1} = 1/a + b$. Since $b < a$, $\tau_{h1} \sim 1/a$ (ref. 34). This rate of channel inactivation, a , was apparently not altered significantly by these mutations. METHODS. The decay phases of K^+ currents evoked by 400-ms depolarizations to various potentials were fit with two exponential time constants, as previously described²², except that the best fit was found by a least-squares fitting procedure. Two factors made it difficult to measure an accurate minimum value for τ_{h1} in mutants which reached their maximum conductance only at very depolarized voltages. First, the conductance of the ShB and mutant channels often decreased at large depolarizations, τ_{h1} sometimes increased as the conductance declined. Second, an endogenous outward current was observed in some batches of oocytes⁴¹. This current activated slowly at voltages above +60 mV; its amplitude was still increasing at the end of a 400 ms depolarization. Because it activated slowly, it did not interfere with determining the peak K^+ current, but it did interfere with measuring τ_{h1} at large depolarizations.



In summary, mutations of basic amino acids in the S4 sequence, as well as of a basic residue in the proposed transmembrane segment H2, affect voltage-dependent properties of the *Shaker* K^+ channel without significantly altering its K^+ selectivity or the kinetic properties of inactivation. These results suggest that the S4 sequence is important in the voltage-dependent activation of the channel. The effects of the mutations cannot be correlated either with their location in the S4 sequence

or with their type (Table 2); these effects may be better understood once there is structural information for the K^+ channel. The deviations of the mutant effects from quantitative predictions of the S4 hypothesis suggest that, in addition to electrostatic interactions between charged residues and the membrane electric field, interactions between the S4 sequence and other parts of the channel, and perhaps cooperativity between subunits, contribute to the sensitivity of the channel to voltage. □

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Spatial distribution of visible lightning on Jupiter

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SPACECRAFT observations have provided evidence for the existence of lightning on Venus¹⁻³, Jupiter^{4,5}, Saturn^{6,7} and Uranus⁸. Little is known, however, about the global distribution of lightning on these planets because of the limited spatial resolution and areal coverage of these previous detections, which have principally involved radio-frequency measurements. Two long-exposure images obtained by the Voyager 1 spacecraft of a small area on the nightside of Jupiter have provided the only previously studied imaging observations of lightning on another planet^{9,10}. Here we present an analysis of all suitable Voyager images of Jupiter and evaluate the horizontal spatial distribution of visible lightning over most of one hemisphere. Essentially all the detectable activity is confined to very narrow latitude bands at 13.5° N and 49° N. The active regions at 49° N are the brightest, most numerous and periodic in longitude. Activity at this latitude is long-lived and is most likely associated with moist convective regions deep in Jupiter's atmosphere. The longitudinal periodicity of the lightning storms may represent the effects of a planetary scale atmospheric wave trapped at the depth of the moist convection^{11,12}.

The Voyager cameras obtained images of Jupiter with high spatial resolution through several broad-band colour filters encompassing the spectral range 0.3–0.7 μm (ref. 4). Image characteristics favourable for the detection of lightning include long-exposure times, low levels of scattered sunlight and adequate spatial resolution to resolve the luminous spots. To evaluate the spatial distribution of the lightning, a pointing reference such as a planetary limb should be visible in each image. We searched for all Voyager images of Jupiter with these

characteristics and found six obtained by Voyager 2 in addition to two previously studied Voyager 1 images^{9,10}. The Voyager 2 observations are particularly well suited to evaluation of the spatial distribution of lightning because most of the nightside hemisphere was sampled with identical camera settings; the Voyager 1 images sampled a much smaller area at high northern latitudes. Although the Voyager 1 and 2 images have comparable exposure times, the spectral range included in the Voyager 2 observations is much narrower. Laboratory measurements of electrical discharges in mixtures simulating the jovian atmosphere show the spectrum of jovian lightning should be dominated by the Balmer spectrum of hydrogen¹³. The three images presented here were taken through the violet filter of the Voyager 2 wide-angle camera which, with an effective wavelength of 0.41 μm , has good sensitivity at the wavelength of the H γ Balmer line¹⁴. The other three images which were taken through an orange filter but which are otherwise identical to the images presented here, had significantly lower spectral sensitivity and thus showed only the brightest features evident in the violet-filter observations. The existence of several images with identical camera settings and little scattered light facilitated accurate removal of background noise due to charged-particle bombardment in Jupiter's magnetosphere and permitted a clear exclusion of camera artefacts, enabling a search for luminous features close to the intrinsic noise level of the instrument.

Figure 1 displays an image of the nightside of Jupiter centred in the northern hemisphere. The bright arc in the image defines the planet's limb and is produced by forward-scattered sunlight from the upper atmosphere. The image has been processed to remove spots and noise associated with the instrument and image transmission to Earth. Several spots exceeding the background noise level over several pixels are evident on the disk of the planet; we interpret them as true luminous events originating on Jupiter. The most striking aspect is the alignment of the brightest features along a line of constant latitude near 50° N planetographic latitude. One feature is evident farther north near the central meridian of the image and two faint features are evident near 15° N latitude. No other features are visible down to the noise level of the instrument. Figure 2 shows a Voyager 2 image of the nightside centred on the equatorial

FIG. 1 A 96-s-exposure image of the nightside of Jupiter taken by Voyager 2 from deep within the planet's shadow on 11 July 1979 at 00:16:44 GMT. The frame is centred on the northern hemisphere and north is toward the top. A latitude-longitude grid with a spacing of 20° has been superposed, and the brightest luminous spots are near 49° N. The image, taken by the wide-angle camera on the spacecraft, includes a spectral range of 0.4–0.45 μm , and the spatial resolution is 100 km pixel⁻¹ at the equator.

METHODS. Each image was produced by first subtracting from the unprocessed digital image of interest another member of the set of six long-exposure images of the nightside. The image-plane coordinates of each group of pixels exceeding the noise level and spanning more than one line were inspected in all of the images to exclude artefacts associated with the imaging system. Figures 1 and 2 include only those spots not excluded by the above procedure. Data numbers in the original images between one and five standard deviations above the noise level of the detector have been stretched to span the full brightness range of the figure; higher data numbers have been included in this figure at their original brightness levels.

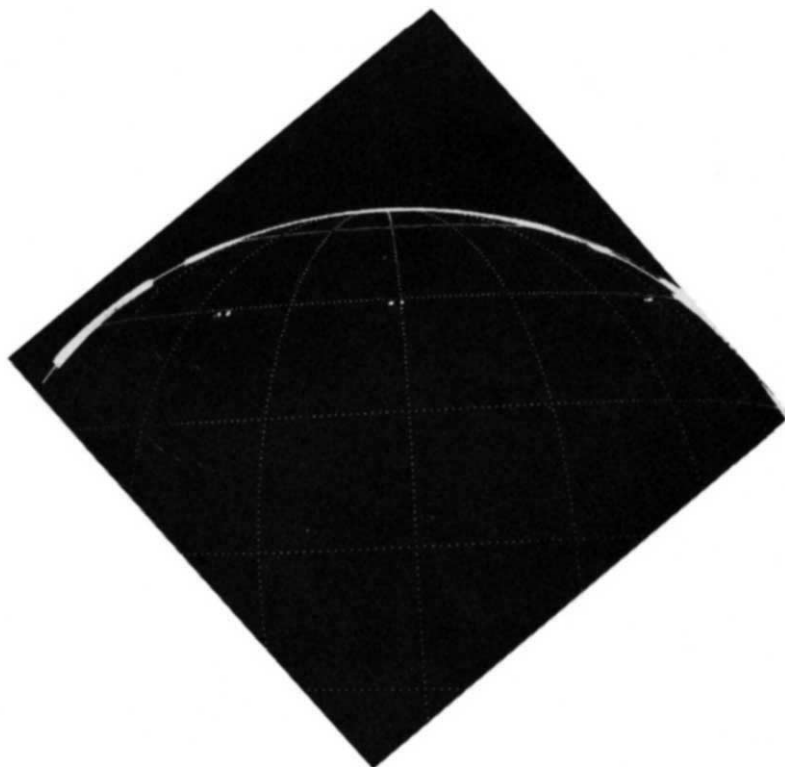
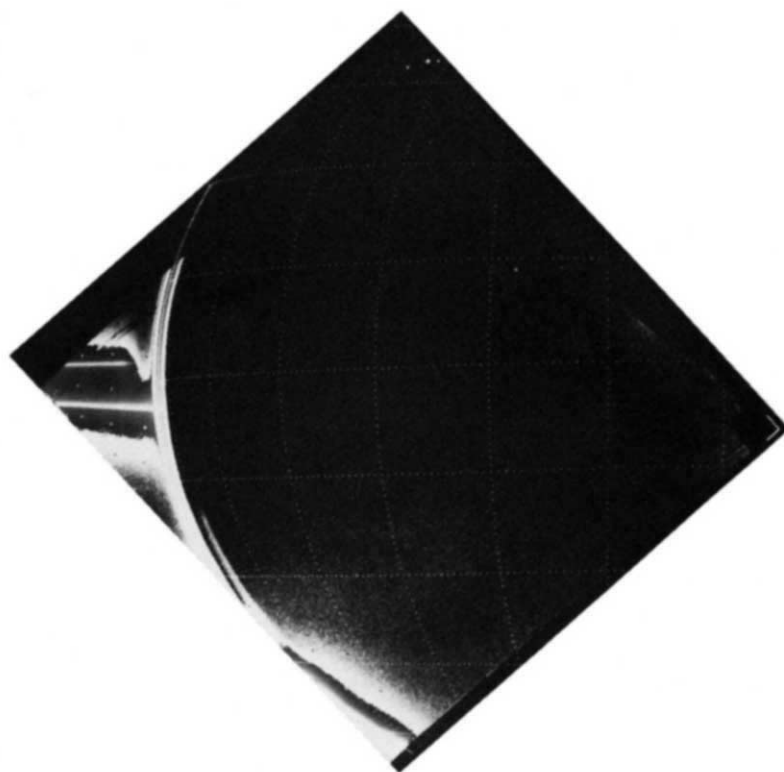


FIG. 2 An image of the nightside of Jupiter centred on the equatorial region and taken within a few minutes of the image in Fig. 1 (at 0:13:32 GMT) with identical camera settings. North is toward the top. A latitude-longitude grid with a spacing of 15° has been superposed and the brightest luminous spots are visible near 49° N. A portion of the jovian ring is visible at the left, and scattered light from the ring is at the lower left. See legend of Fig. 1 for further explanation.



region. The bright luminous features at the top of the image are near 50° N latitude, and one faint feature near 15° N is again evident.

The positions of the luminous spots identified in Figs 1 and 2 and in a third violet-filter image are displayed on a rectangular projection map in Fig. 3, along with outlines of the areas sampled by each image. The spots show a striking latitudinal confinement. Fourteen of the eighteen features observed are between 49° N and 50° N latitude to within the uncertainties ($\sim \pm 0.5^\circ$ in latitude). Wherever the images overlap, features are seen at the same locations in each image to within the positional uncertainties. This repeatability excludes meteors as a plausible source for the luminous spots. Alignment of the features with lines of planetographic latitude excludes interaction between the magnetosphere and atmosphere as a plausible source for the spots because the magnetic field is significantly tilted with respect to the rotational pole of the planet¹⁵. We conclude that the observed features are most plausibly interpreted as lightning discharges in the atmosphere of Jupiter.

The observations presented here have allowed most of one hemisphere to be searched with uniform sensitivity for highly energetic visible-lightning features. Assuming that the observed emission is the $H\gamma$ Balmer line, the observed energies from this line range from 5×10^8 to 10^{10} J with the highest energies occurring at 49° N. If the full spectrum of the discharge is given by the Balmer spectrum of hydrogen¹³, the observed energies correspond to total optical energies of 2×10^{10} – 4×10^{11} J per feature. These energies are somewhat greater than the optical energies reported in the Voyager 1 images¹⁰ and are significantly larger than the optical energies radiated by ordinary terrestrial lightning ($\sim 10^7$ – 10^8 J)^{10,16} and terrestrial superbolts ($\sim 10^9$ J)¹⁷ over the same interval of time. Figure 3 shows that the high-energy events on Jupiter show strong latitudinal confinement and that the events at 49° N latitude are periodic in longitude. Comparison with the Voyager 1 observations, which are also displayed in Fig. 3 and which were obtained about four months earlier, shows that lightning activity was also occurring at 49° N at the time of the Voyager 1 encounter. The longitude of the most active centre in the Voyager 1 observations is consistent

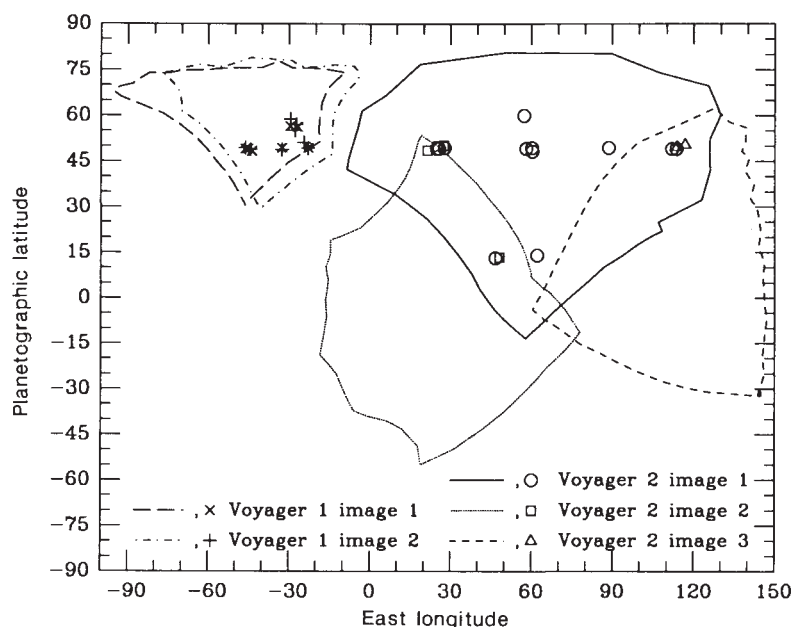
with an extrapolation to the west of the periodicity seen in the Voyager 2 results. Such narrow latitudinal confinement and a planetary-scale periodicity are not evident in terrestrial lightning activity, which correlates well with the distribution of land mass and varies strongly with season¹⁸.

Lightning activity in the Earth's atmosphere is generally associated with large-scale charge separation in thunderclouds¹⁹, and satellite observations of the global distribution of midnight lightning activity show that it correlates with the distribution of tropospheric convective activity¹⁸. The deep atmospheres of the giant planets are inferred to be convective up to about the base of the visible clouds (at a pressure of ~ 700 mbar)²⁰. The condensation of water and other heavy constituents in the principally hydrogen-helium atmospheres of the giant planets produces initially negative buoyancy contrasts which must be overcome to initiate moist convection and makes the prediction of its horizontal spatial distribution difficult^{21,22}. Modelling of the transfer of radiation from a point discharge in the jovian atmosphere indicates that the intensity profiles of the Voyager 1 lightning features at 49° N can be best understood as scattered radiation from a lightning discharge at a pressure of ~ 5 bars, which corresponds to the level at which water condenses, if oxygen is present in solar abundance²³. Figure 3 may thus be providing a map of regions of strong moist convection at depth in Jupiter's atmosphere. The regular spacing in longitude of the clusters of lightning at 49° N is consistent with a planetary wavenumber of 12. Vertical trapping of planetary waves in a stably stratified duct at the water-cloud level has been proposed as an explanation for the prominent and periodic cloud plumes on Jupiter¹², which are at $\sim 5^\circ$ N and have a wavenumber 11–13. The striking agreement between the characteristics of this model and of the lightning at 49° N suggests that extending the use of the idea of a trapped planetary wave to higher latitudes may explain the longitudinal periodicity of the lightning. The wavenumbers of the lightning at 49° N and of the plumes are similar, but we do not see evidence of visible lightning at the latitude of the plumes.

Absorption and scattering by aerosols could be biasing the observed distribution of lightning activity. We have used a

FIG. 3 Map of positions of luminous spots and outline of areas included in the Voyager 2 nightside images. Images 1 and 2 are displayed in Figs 1 and 2, respectively. Image 3 was taken a few minutes earlier at 00:03:56 GMT with identical camera settings. The uncertainties in the positions are $\sim 0.5^\circ$ in latitude and $\leq 3^\circ$ in longitude. In regions of overlapping image coverage, luminous spots are visible at the same planetographic coordinates to within the uncertainties. Results from the two Voyager 1 images of lightning, which were obtained on 5 March 1979, are also displayed and have positional uncertainties comparable to the Voyager 2 results; the exposure time of each image was 160 s.

METHODS. The conversion of image-plane coordinates to planetographic coordinates was determined by using the limb of the planet as a pointing reference. Forward-scattered light from the upper atmosphere defines the limb in the Voyager 2 images, and the aurora was used to define the limb in the Voyager 1 images. Translational and rotational motion of the spacecraft during the long-exposure times determines the width of the bright limb or aurora and governs the uncertainty of the planetographic coordinates. The positional uncertainties have been estimated by evaluating positions based on the inner and outer edges of the broadened limb.



Monte Carlo radiative-transfer program²³ to calculate the amount of aerosol needed to attenuate a point source with the luminosity of the brightest features in the Voyager 2 images to the noise level of the camera. Using previously estimated values for the properties of aerosols in the upper troposphere of Jupiter²⁴, we find that normal aerosol optical depths in excess of 30 would be required. Such large aerosol optical depths extending over large areas of the planet seem to be excluded above the 2-bar pressure level of the atmosphere based on ground-based studies of sunlight reflected from the planet²⁴. Therefore, we cannot exclude the possibility that significant aerosol optical depths are masking some of the lightning activity on Jupiter. If this is the case, however, the masking must be due either to significant previously undetected widespread opacity well below the visible clouds or due to compact optically thick clouds associated with the moist convection itself.

The stability of the latitudes of lightning activity at 49° N over the time between the Voyager spacecraft encounters and over a wide range of longitudes is striking. This stability is far greater than that of lightning on the Earth¹⁸ and of most of the visible cloud features on Jupiter^{20,25}. But Jupiter's east-west wind system^{20,25}, the Great Red Spot²⁵, and recently discovered large-scale slowly moving thermal structures²⁶ all show a similar lack of variability. The observed lightning activity seems to be probing a moist convective dynamical regime at depth which shows large-scale organization and long dynamical timescales. Future imaging observations of lightning by orbiting spacecraft may be useful for investigating the deep convective regimes of the giant planets. □

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A non-porous supported-platinum catalyst for aromatization of *n*-hexane

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THE cyclization of C_6 and C_7 *n*-alkanes to form aromatic compounds is highly desirable in the petroleum-refining industry. Platinum metal clusters incorporated into the channels of zeolite L have been found to catalyse the aromatization of hexane and heptane with high activity and selectivity^{1,2}. The susceptibility of this catalyst to sulphur poisoning, however, means that the hydrocarbon reagents must be of high purity, free from sulphur contamination². It has been generally believed that the pore structure of the zeolite plays an important part in the selectivity of the catalytic process. Here we describe a non-porous platinum catalyst that can convert *n*-hexane to benzene with an activity and selectivity comparable to that of the zeolite. The catalyst consists of platinum clusters, about 2 nm in diameter, supported on a basic, high-surface-area magnesium oxide stabilized by aluminium. The product distribution for *n*-hexane is almost identical to that obtained using the zeolite L catalyst. These results point to a new approach

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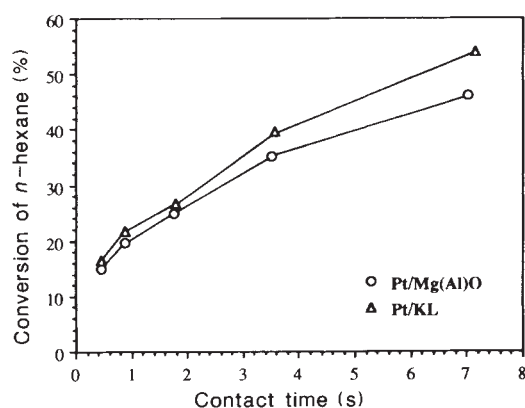


FIG. 1 Comparison of magnesia- and zeolite-based Pt catalysts for *n*-hexane conversion at 750 K, atmospheric pressure, and a H_2 /hydrocarbon—molar ratio of six.

to hydrocarbon reforming based on metal species supported on highly basic, non-porous carriers.

Conventional hydrocarbon-reforming catalysts are composed mainly of noble-metal clusters supported on an acidic carrier such as alumina and are not effective for the aromatization of *n*-alkanes. These catalysts operate through a bifunctional mechanism in which the noble metal provides a dehydrogenation/hydrogenation site and the acidic support catalyses structural rearrangements of the hydrocarbon chain³. Research with alkali-exchanged zeolites has produced an active and selective commercial catalyst, consisting of Pt clusters supported in the one-dimensional channel structure of zeolite L^{1,2}, for the conversion of C_6 and C_7 hydrocarbons to aromatics. The non-acidity of the zeolite and correlation of reaction rate with Pt loading indicate that the catalytic function of this catalyst depends only on the Pt metal. We have studied the dehydrocyclization of *n*-hexane over a MgO catalyst, the basicity of which may also lead to a monofunctional reaction mechanism.

We prepared stabilized high-surface-area MgO, according to the methods described in ref. 4, by coprecipitation of magnesium and aluminium nitrate salts in a 5:1 Mg:Al molar ratio. Nitrates were used rather than sulphates to avoid sulphur poisoning of the catalyst. The resulting hydroxycarbonate (hydrotalcite structure by X-ray diffraction) is a convenient precursor to a high-surface-area Mg(Al)O that is heat and steam stable. The presence of Al seems to have an important role for this purpose and has no detrimental effect on the pronounced basic character of the 'stabilized' magnesia⁴. Following decomposition in air at 873 K for 12 h, the specific surface area of the final stabilized magnesia, as determined by N_2 adsorption, was $220\text{ m}^2\text{ g}^{-1}$. We found no evidence for a zeolite-type microporosity in the N_2 sorption isotherm obtained at very low relative pressure ($10^{-5} < p/p_0 < 10^{-1}$). The support-stabilized magnesia was impregnated with an aqueous solution of $Pt(NH_3)_4Cl_2$, dried, and calcined in air at 650 K for 6 h to give a sample with 0.88 wt% Pt. After reduction in H_2 at 700 K for 2 h, the sample Pt/Mg(Al)O had a fraction of Pt metal atoms exposed to the surface of 0.49 as determined by H_2 chemisorption, which corresponds to an average cluster diameter of $\sim 2\text{ nm}$.

For comparison, we also prepared the potassium form of the zeolite L catalyst, Pt/KL. This was ion-exchanged with aqueous $Pt(NH_3)_4Cl_2$, dried, calcined and reduced according to the above procedure to give a final Pt loading of 0.52 wt%. The fraction of Pt metal atoms exposed to the surface was found to be 1.0 by H_2 chemisorption, which corresponds to metal particles of $\sim 1\text{ nm}$ average diameter. Metal clusters of this size are small enough to fit inside the cages of zeolite L^{1,2,5}.

We tested the steady-state conversion of *n*-hexane by both samples in a single-pass tubular reactor at 750 K and atmos-

TABLE 1 Product distribution near 40% conversion

	Pt/Mg(Al)O	Pt/KL
Conversion %:	35.1	39.6
Selectivity %:		
Benzene	43.7	43.9
Methylcyclopentane	11.2	9.3
C_6 Isomers*	5.1	4.9
Hexenes†	17.4	16.1
Others‡	22.6	25.8

Conditions: H_2 /hexane = 6; atmospheric total pressure, 750 K; diluent He.

* 2- and 3-methylpentanes, 2,2-dimethylbutane.

† 1- and 2(cis, trans)-hexenes. 3-hexenes could not be separated from hexane.

‡ C_1 – C_5 hydrocarbons, dienes and so on.

pheric pressure (6 kPa hydrocarbon, diluent He), with a molar H_2 : hydrocarbon ratio of six. Contact times were calculated from the flow rate of *n*-hexane and the quantity of Pt surface atoms in the reactor. A typical run consisted of loading $\sim 300\text{ mg}$ of catalyst (pressed, crushed and sieved to 0.25–0.50 mm grains) into the reactor and re-reducing the Pt *in situ* with flowing H_2 at 700 K for 2 h before introducing reactant *n*-hexane. For proper comparison, the number of surface Pt atoms in the reactor was the same for each run and all the results reported below were obtained after at least 1 h on stream.

Figure 1 shows the conversion of *n*-hexane with increasing contact time for the Pt/Mg(Al)O and Pt/KL samples. The conversion is nearly the same for the two catalysts regardless of contact time, demonstrating the similarity in catalyst activity per surface Pt atom. Even more striking is the comparison in Fig. 2. The selectivity toward the desired product, benzene, is virtually identical for both catalysts at the levels of conversion studied. The product distribution at $\sim 40\%$ conversion for the two catalysts is given in Table 1. The catalysts exhibit similar selectivity for the products formed by aromatization, cyclization, isomerization and dehydrogenation. For the Pt/Mg(Al)O catalyst, runs of up to 40 hours showed that constant selectivities were maintained at constant conversion level.

The low production of hexane isomers in both cases suggests the lack of significant acidity of the support magnesia and zeolite. Previous work verified that incorporation of aluminium does not alter the pronounced basicity of magnesium oxides⁴. Reduction of Pt^{2+} cations in the zeolite by hydrogen, however, induces residual acidity. We have therefore ion-exchanged a previously reduced Pt/KL zeolite with aqueous Ba^{2+} to remove any acidity resulting from the reduction process. Barium was used because

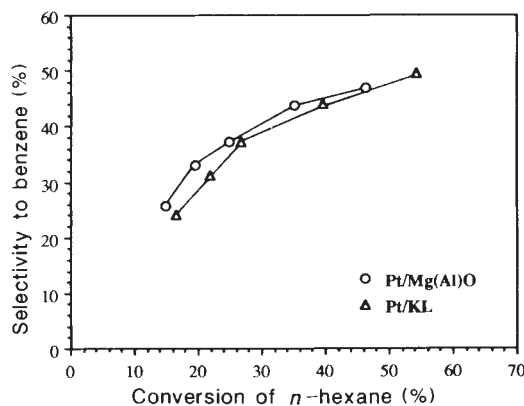


FIG. 2 Selectivity to benzene formation during *n*-hexane reaction over Pt catalysts at 750 K, atmospheric pressure, and a H_2 /hydrocarbon molar ratio of six.

the large cation increases the basicity of zeolite L, and because a Pt/Ba zeolite L has been reported⁶ to be an excellent catalyst for aromatization of *n*-hexane. We observed no improvement in the catalyst performance after this ion exchange. Therefore, the comparison of Pt/Mg(Al)O to Pt/KL seems to be unaffected by any residual acidity introduced by reduction.

The remarkable activity and selectivity of the magnesia-based catalyst is totally unexpected, because zeolite-based materials are superior to conventional non-zeolite Pt catalysts¹. Earlier work with Pt-zeolite-based materials (mordenite, X, Y, Ω and L) showed that zeolite L was the best support for aromatization of *n*-hexane¹. A molecular graphics study of hydrocarbon interactions with a Pt cluster inside the channel of zeolite L revealed that *n*-hexane has adequate space to curve around and attach both ends to a single Pt atom⁷. It was proposed that the spatial limitations in the zeolite L channel maximize the van der Waals interactions of the curved hydrocarbon with the channel walls leading to a stabilized cyclic intermediate. The fact that Pt clusters in zeolites L and Y were accessible only through pores of ~ 0.73 nm defined by their cage windows was also proposed to be a critical feature⁵. Finally, the influence of the zeolite environment on the atomic and electronic structure of the Pt clusters may contribute to the observed catalysis. Metal-support interactions for the Pt/zeolite L system have recently been identified⁸. Therefore, no single reason can be given for the unusual activity and selectivity for Pt/zeolite L catalysts.

For the Pt/Mg(Al)O system, no channel or cage structure analogous to a zeolite exists. We can therefore rule out any influence of spatial constraints on the stabilization of a cyclic

intermediate. One possible explanation for the observed catalysis is a modification of the atomic and electronic structure of the Pt clusters through a metal-support interaction in a way that optimizes the production of aromatics. Because the support magnesia is highly basic, another plausible explanation is that the metal-catalysed aromatization reaction is favoured over unwanted isomerization or hydrogenolysis side reactions known to occur on acid sites. Therefore, the support magnesia may act only as an inert carrier for the Pt clusters. A third possibility would be a bifunctional mechanism in which basic sites on the magnesia support supplement the catalytic sites of the Pt clusters.

Our work demonstrates the potential usefulness of high-surface-area non-zeolite basic supports for hydrocarbon-reforming reactions. We are now investigating further the physical properties, stability and resistance to sulphur poisoning of Pt/Mg(Al)O materials. $\square$

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Crystallization of an inorganic phase controlled by a polymer matrix

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BIOLOGICAL composite materials such as bones, teeth and shells consist of a polymer matrix reinforced by an inorganic phase which forms in the matrix¹. These materials are distinguished from synthetic composites by the high degree of organization and regularity displayed by the inorganic phase: inorganic minerals of uniform size, morphology and crystallographic orientation can be formed in ordered arrays in living cells. Such a process has until now not been realized in synthetic systems, although the recent interest in nanoscience^{2–6} has stimulated much research in the area. We report here an example of a synthetic process that produces composite materials analogous to those produced by natural biomineralization. The inorganic/organic *in situ* synthesized composites display controlled inorganic crystal size, morphology and orientation, which are determining features of type II, or matrix-mediated⁷, biocomposites. The synthetic factors that must be optimized to give biomimetic properties to synthetic composites are strong binding of the inorganic reagents by the organic matrix (molecular complementarity); good 'solvation' of the inorganic reagents by the polymer; and an ordered, regular polymer environment in which to induce nucleation (matrix preorganization)¹.

In designing a process to synthesize composites with the same desirable characteristics as biologically synthesized materials, we chose *in situ* synthesis as the method that most closely approaches biological processes⁷. This method is not new, but without attention to the aforementioned factors does not give

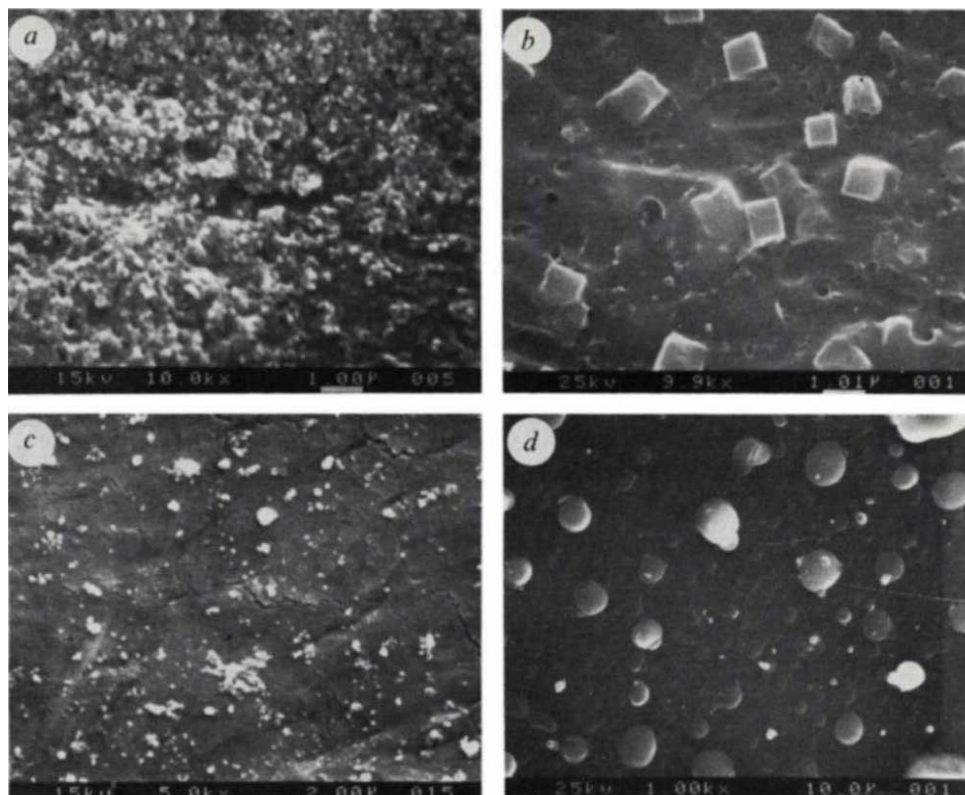
composite products with controlled physical properties. We chose CdS as the inorganic phase to be synthesized in preliminary experiments because of its potential importance as a nonlinear optical material⁸, the ease of its synthesis⁹, its particle-size-dependent electronic spectrum¹⁰, and the many reports on nanoscale CdS particles^{11–13}. To mimic biosynthetic mechanisms as closely as possible, poly(ethyleneoxide) (PEO) was chosen as the polymer matrix. This polymer binds metal ions strongly, forming crystalline complexes¹⁴, and thus more closely resembles biological systems in which inorganic ions interact strongly with an organic matrix¹.

In a typical experiment, the solution formed by dissolving PEO (0.45 g) and CdCl₂ (0.05 g, 10% by weight) in 10 ml water was dip-coated onto a glass microscope slide, forming a film of PEO/CdCl₂ ~ 0.2 mm thick. The X-ray diffraction patterns of the films show the characteristic lines of crystalline PEO as well as new peaks arising from the formation of PEO-Cd crystalline complexes¹⁴. The film was then removed from the slide and immersed in a solution of tetrahydrofuran containing 0.02 M S(Si(CH₃)₃)₂. After 10 days, reaction to form polymer-solvated CdS is complete (equation (1)), as shown by the presence of Cd and S and the absence of Cl and Si in the X-ray fluorescence spectrum of the rinsed composite film



The presence of CdS is confirmed by the electronic spectrum of the film, which shows in this case the characteristic absorption of the bulk semiconductor. Scanning electron microscopy (SEM) shows the spongy surface of the film (Fig. 1a), but no definite CdS particles can be detected. X-ray fluorescence of a cross-section of the film shows CdS evenly distributed throughout, and X-ray diffraction of the composite indicates that the CdS is amorphous. When the CdS/PEO composite film is heated in an inert atmosphere to 120 °C for 1 hour, then cooled, its electronic absorption band shifts towards the red, and peaks characteristic of both common forms of CdS, the wurtzite and the zincblende structures, appear in the diffraction pattern. SEM reveals that large (1 μm on an edge) regular cubic

FIG. 1 *a*, Scanning electron micrograph of a polyethyleneoxide film after *in situ* synthesis of CdS at room temperature. *b*, Same film after heating to 120 °C for 1 h. *c*, Scanning electron micrograph of polyethyleneoxide film after *in situ* synthesis of CdS at 120 °C. *d*, Scanning electron micrograph of polymethylmethacrylate film after *in situ* synthesis of CdS at 120 °C.



crystals of CdS have formed (Fig. 1*b*), presumably by the ordered aggregation of smaller particles formed during the room-temperature reaction. Such ordered aggregation of mineral particles is postulated to be one of the processes by which natural biomineralization may occur¹⁵. In confirmation of this mechanism, the direct reaction of a PEO/10% CdCl_2 film with $\text{S}(\text{Si}(\text{CH}_3)_3)_2$ in octane at 120 °C gives CdS crystalline product of merely random morphology (Fig. 1*c*), and reaction of CdCl_2 in an amorphous polymer such as poly(methylmethacrylate) (PMMA) (cast from an acetone–water solvent) with $\text{S}(\text{Si}(\text{CH}_3)_3)_2$ gives spherical crystalline particles (Fig. 1*d*). In these syntheses, the organic polymer matrix mediates the inorganic synthetic reaction and determines the morphology of the product. Regular crystals of controlled size and habit are not formed unless the initial nucleation takes place in an ordered, crystalline environment that binds the reagents well. Heating

the polymer matrix above its melting point (m.p. of PEO is 67 °C) destroys the crystallinity of the matrix and therefore its preorganization, and only random-morphology product is produced (Fig. 1*c*). However, if the initial particles are formed in the ordered polymer matrix, they agglomerate upon heating into larger crystals of regular size and habit.

In order to determine better the size and morphology of the initially synthesized room-temperature particles, films of PEO/10% CdCl_2 ~150 nm thick were dip-coated directly on nickel transmission electron microscopy (TEM) sample grids. The grids were then immersed in 0.02 M $\text{S}(\text{Si}(\text{CH}_3)_3)_2$ -octane solutions at room temperature for 7 days (complete reaction). TEM showed CdS aggregates ~500 nm in diameter (Fig. 2*a*) which were amorphous by electron diffraction. These can be regarded as models for the initial particles formed at room temperature in the bulk film. By making the $\text{S}(\text{Si}(\text{CH}_3)_3)_2$ -octane

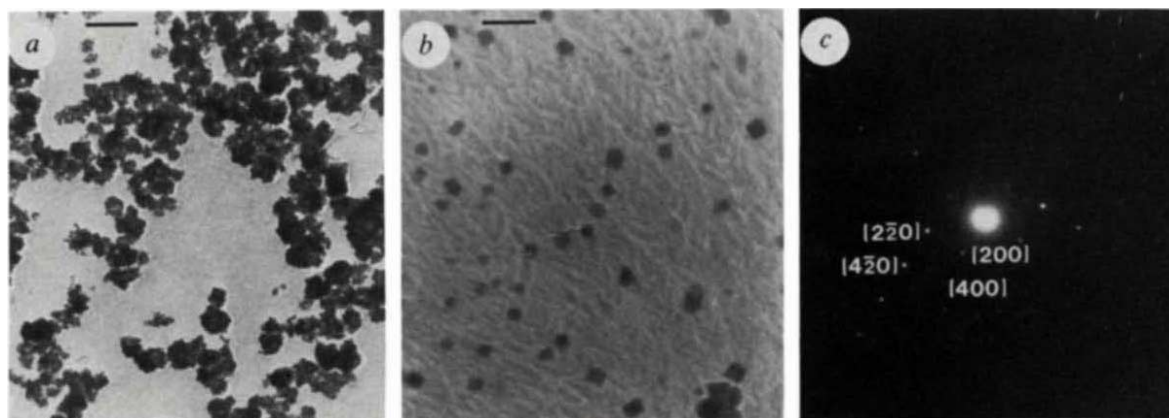


FIG. 2 *a*, Transmission electron micrograph of polyethylene oxide film after *in situ* synthesis of CdS at room temperature. Scale bar, 1,000 nm. *b*, Transmission electron micrograph of polyethylene oxide film after *in situ*

synthesis of CdS at room temperature in the presence of AOT. Scale bar, 1,000 nm. *c*, Single-crystal electron diffraction pattern of a crystal in the film shown in *b*. Camera length, 69.7 cm.

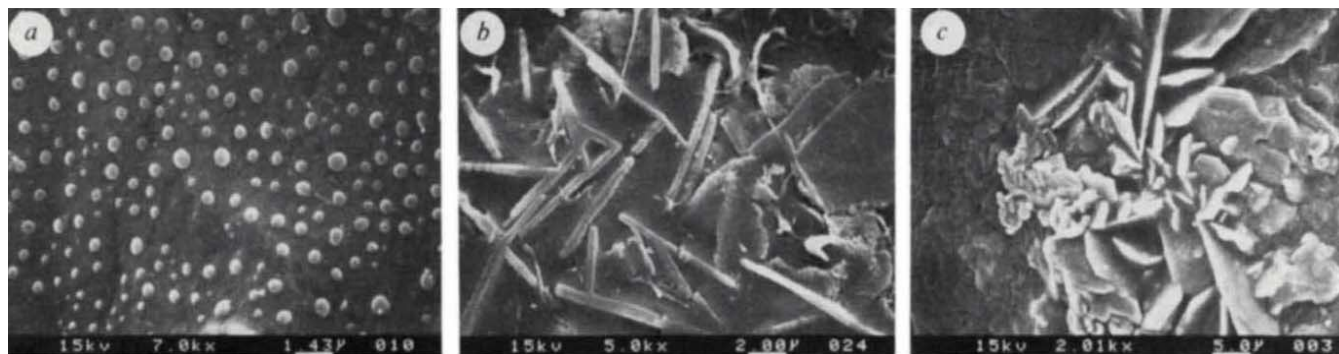


FIG. 3 *a*, Scanning electron micrograph of a polyethylene oxide film after *in situ* synthesis of CdS at 120 °C and 6 h annealing at 200 °C. *b*, Scanning electron micrograph of a polyethylene oxide film after *in situ* synthesis of

CdS using the same conditions as *a*, but the presence of 0.06 M 2,2',2''-tri-aminotriethylamine. *c*, Scanning electron micrograph of a polyethylene oxide film after *in situ* synthesis of CdS at 56 °C.

solution 0.03 M in the surfactant sodium di(2-ethylhexyl)-sulphosuccinate (AOT) an even more ordered environment can be induced in the semicrystalline polymer matrix. The room-temperature CdS product is considerably altered. A few crystals of bipyramidal habit, of the order of 1 μm on an edge, are formed. The number of these crystals depends strongly on the AOT concentration, and reducing the concentration greatly reduces their number. In all cases, however, many cubic single crystals, ~ 200 nm on an edge, were also formed throughout the film (Fig. 2*b*). The crystals were orientated such that all displayed the allowed *hk*0 reflections in their electron diffraction patterns, but we saw no traces of the allowed reflections with $l \neq 0$. The crystals were thus uniformly aligned with a crystallographic fourfold axis (the [001] zone) perpendicular to the plane of the organic film (Fig. 2*c*). Also, single-crystal electron-diffraction patterns of many individual crystals, as well as powder electron-diffraction patterns of different areas of the films containing many crystals, indicate that the crystals had the rock-salt structure (Fig. 2*c*), a phase previously seen in CdS only at high pressures¹⁶. A recent study of CdS particles synthesized *in vivo* by yeast cells also indicated that the biomineralized particles may adopt this structure^{17,18}. In our case, the AOT could assemble on the film's surface with its polar sulphonate head group interacting with the polar PEO, and the non-polar alkyl tail extending into the octane solution. Selective crystallization and orientation could then be mediated by the head group's interactions with polymer-bound Cd^{2+} ions, as was seen in crystallizations from solutions with a Langmuir-Blodgett layer of surfactant on the air/liquid interface^{19,20}.

Besides matrix mediation of nucleation, two other key features of biomineralization are control of ion flux at the matrix interface, and growth and habit modification by soluble molecules within the matrix¹⁵. We see analogous effects in this synthetic system. Particle or crystal size of the CdS in the PEO film can be varied by altering the initial CdCl_2 concentration: we have obtained particle sizes from 2 nm to 2 μm . Crystal habit is similarly altered by addition of ligands. For example, the spherical morphology found in 8% CdS/PEO synthesized at 120 °C and annealed at 200 °C for 6 h (Fig. 3*a*) changes to a fibrous one when the reaction occurs in the presence of 0.06 M 2,2',2''-tri-aminotriethylamine (Fig. 3*b*). These fibres closely resemble the ordered chains of magnetite biosynthesized in magnetotactic bacteria, as they are also composed of small crystallites linearly arranged²¹. Use of a different crystalline polymer matrix also alters the CdS product morphology (Fig. 3*c*).

The control of the inorganic phase's size, morphology, crystalline phase and crystallographic orientation that this biomimetic synthesis allows would be highly desirable in fabricating composite materials with technological applications^{1,7,22}. We

are now applying this synthetic method to the fabrication of other inorganic materials in ordered arrays within polymer matrices. $\square$

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Shock-induced martensitic phase transformation of oriented graphite to diamond

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ONE important method of diamond synthesis is shock compression of graphite and other forms of carbon to high pressures and temperatures, and subsequent quenching to yield metastable diamond. This process, which occurs in microseconds, happens naturally in the impact of meteors^{1,2}, within products of explosives^{3,4}, and by explosive compression of powders^{5,6}. A major unresolved issue is whether the shock-induced phase transition of graphite to diamond is martensitic or diffusive. The relation between the crystal structures of graphite and hexagonal diamond suggests that the phase transition should be fast and martensitic if shock pressure is applied parallel to the *c* axis (normal to the basal planes) of the graphite crystal structure. Here we report measurements of shock-wave histories for this transition which

show that it occurs in ~ 10 ns. These results imply that the transformation from graphite to diamond is martensitic for temperatures substantially lower than the melting temperature. We observe an unexpectedly large sensitivity of kinetics to sample morphology. As well as answering questions concerning the physical nature of the transformation, our results are relevant to optimization of diamond yield in industrial synthetic methods.

Shock⁶⁻⁸ and static⁹ high-pressure synthesis of diamond from graphite produces hexagonal (lonsdaleite) and cubic diamond. Higher temperatures increase the yield of the cubic phase⁷⁻⁹. Comparison of the relative crystallographic orientations of product and reactant phases of shock-synthesized diamond particles provides evidence for a martensitic transformation from hexagonal graphite to hexagonal diamond⁷ for temperatures $< 2,000$ K, which are low compared with the melting temperature of carbon ($\sim 4,000$ K at ~ 20 GPa)⁹. For temperatures of 3,000–4,000 K, only cubic diamond is observed, regardless of the initial carbon phase, indicating that the transition is reconstructive and diffusive at these temperatures. At intermediate temperatures, in which a mixture of the two phases is produced, there is some microstructural evidence that shock synthesis of cubic diamond from graphite has two steps: a martensitic transformation to lonsdaleite, followed by a second transformation, possibly martensitic, from the hexagonal to the cubic phase⁷. Thus these microstructural results indicate that the phases produced are determined by the transformation mechanism, which in turn is strongly dependent on temperature.

A major unresolved issue is why, if the transition from graphite to hexagonal diamond is martensitic, the transformation has not been observed in real-time shock experiments with nanosecond time resolution. As systematic atomic displacements take hexagonal graphite into hexagonal diamond, this transformation is expected to be martensitic and therefore should happen on application of shock pressure over a timescale of a few nanoseconds. Observation of a fast transformation would provide definitive confirmation of a martensitic mechanism. Time-resolved observations may also provide clues for technological improvements such as optimizing the efficiency of transforming graphite and retaining diamond.

Although real-time shock-compression experiments have been performed previously, interpretation of these data is not unambiguous, largely because of the initial microstructures of the graphite specimens: the experiments were performed with pyrolytic and porous graphite. Pyrolytic graphite is a quasi-single-crystal form with the basal plane of the hexagonal crystal structure oriented preferentially in the plane of the constituent platelet grains, such that the specimens have a mosaic structure with the *c* axis of each crystallite distributed a few degrees about the specimen normal. Misalignment of crystallites in the mosaic structure may exert a heterogeneous influence on the transformation mechanism. Porous graphite is a compact of powder particles with porosity up to a few tens per cent. When porous material is shock-compressed, material immediately around collapsed pores is shock-heated to much higher temperatures than the material further from the pores¹⁰, with the result that phase-transformation studies of porous graphite are complicated by spatially heterogeneous temperature distributions and thus again by possible spatial distributions of the transformation mechanism.

A martensitic transformation should yield a well defined onset pressure P_{0t} of the transition. Previous shock-wave equation-of-state data¹¹⁻¹⁵ for pyrolytic and porous graphite, however, show a range of P_{0t} , with porous samples exhibiting a lower P_{0t} (~ 20 GPa) than dense pyrolytic ones. This variation in P_{0t} has been attributed¹⁶ to a thermally activated diffusive (rather than martensitic) transformation driven by higher shock temperatures in porous specimens.

The most direct method of observing shock-induced phase transitions is to measure time histories of shock waves. Because the graphite-to-diamond transition involves a relatively large

volume change, the shock-wave history should show two steps (a 'two-wave' structure) in shock pressure or material velocity¹⁷. The first step corresponds to shock compression of graphite to P_{0t} , and the second corresponds to a rise in pressure to the phase-transformed state. Shear stress, which exists in shocked solids, is expected to drive this transition quickly by inducing displacive shear motion between lattice planes so as to produce the hexagonal-diamond stacking sequence. Thus for martensitic transitions, the second rise should be rapid, lasting perhaps several nanoseconds. A two-wave structure has been observed in porous graphite¹⁸, but because of the heterogeneous nature of shock heating in such specimens, these results are not definitive in determining the transformation mechanism.

We shock-compressed highly oriented pyrolytic graphite parallel to the *c* axis and measured the shock-wave velocity profile. Shock waves were generated by impact of a copper disk accelerated by a two-stage light-gas gun to velocities of 2.6–3.4 km s^{-1} . The copper disk struck an aluminium baseplate on which a graphite specimen was mounted. The specimen was backed by a LiF window through which a laser beam was reflected off the rear surface of the graphite. As LiF has a shock impedance close to that of graphite, the perturbation of the shock wave by the interface is relatively small. The laser is the probe beam of our VISAR interferometer¹⁹, which measures the velocity of the graphite–LiF interface to 0.1% with a time resolution of 2–3 ns.

Two grades of pyrolytic graphite (ZYG and ZYH) obtained from Union Carbide were used. The specimens were 2.5 mm thick and at least 13 mm square. The grades are distinguished by the value of β , the angular width of the (002) X-ray diffraction peak, which measures the spread of misalignment of the crystallite *c* axes. For the high- and low-grade samples β is 0.8° and 3.5° respectively. The high-grade samples had a shiny metallic lustre, and the low-grade ones had a dull 'pencil-lead' appearance. The densities were 2.254 and 2.262 g cm^{-3} for the high and low grades, respectively, which are very near the handbook value²⁰ of 2.265 g cm^{-3} .

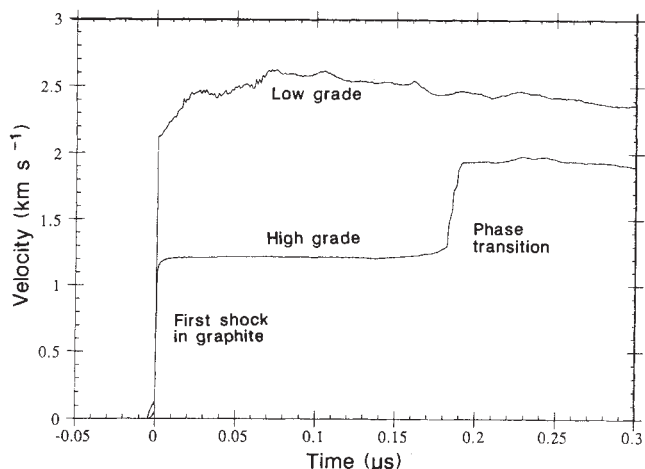


FIG. 1 Velocity profiles of pyrolytic graphite shocked parallel to the *c* axis of the graphite crystal structure and measured at the graphite–LiF interface for high-grade (shot g2) and low-grade (shot g5) samples. Target g2 was impacted by a 3-mm-thick copper disk at 2.69 km s^{-1} , and target g5 by a 2-mm-thick disk at 3.57 km s^{-1} . The high-grade sample shows a distinct two-wave structure. The first wave indicates the shock compression of graphite to 21 GPa. The second wave indicates a rapid (~ 10 ns) martensitic transformation to a diamond-like density. The time separation between the two waves is not caused by time latency of the transformation, but by the slower speed of the second wave through the material owing to the increased compressibility at the phase transition. The low-grade sample shows a frustrated transition over several tens of nanoseconds. The final density is significantly less than that of diamond at the same shock pressure, suggesting a complex intermediate state.

Three shots of each grade were fired. Figure 1 shows velocity histories for high- and low-grade samples. The two-wave nature of the high-grade shot is direct evidence for a fast transformation. In the first wave the graphite is shock-compressed to P_{01} , which is calculated through standard analysis to be 21 GPa (20 and 22 GPa for the other two shots). In the second wave the graphite is compressed in the transformation to a denser phase, calculated to have density $\rho = 3.63 \text{ g cm}^{-3}$, at a pressure $P = 30 \text{ GPa}$. Comparison to the density of shocked diamond²¹ ($\rho = 3.72 \text{ g cm}^{-3}$ at 30 GPa) suggests that transformation to diamond was nearly complete. The rapid rise ($\sim 10 \text{ ns}$) of the second wave in the high-grade sample is evidence for a martensitic transition. The estimated shock temperature of 1,800 K, calculated below, is in the stability field of hexagonal diamond⁹.

The wave history of the low-grade sample shows relatively sluggish kinetics, a higher velocity at the onset of the transition, and thus a greater P_{01} of 42 GPa (31 and 24 GPa for the other shots.) Its second shock state, $\rho = 3.36 \text{ g cm}^{-3}$ at $P = 49 \text{ GPa}$, is significantly less dense than diamond ($\rho \approx 3.8 \text{ g cm}^{-3}$) at 49 GPa. The second-shock state is probably a dense, disordered phase. Evidently, the phase-change behaviour of pyrolytic graphite is very sensitive to the degree of orientational order in the specimen.

The temperature of crystal-density graphite shocked to $P_{01} = 21 \pm 1 \text{ GPa}$ was calculated to be $T \approx 500\text{--}550 \text{ K}$ by integrating $dT = (V_0 - V)dP/2C_v + [P/2C_v - T\gamma/V]dV$ along the graphite pressure-volume shock adiabat^{14,22}. Here $C_v(T)$, the heat capacity, is taken from ref. 23. The Grüneisen γ was assumed to range between its graphite and diamond values²⁴, $\gamma = 0.35$ and $\gamma = 1.15$ respectively. The temperature of the second shocked state for the high-grade sample was estimated to be $T \approx 1,800 \text{ K}$ via the relation $\Delta T = \Delta P/(\rho\gamma C_v)$, where $\Delta P \approx 13 \text{ GPa}$ is the thermal pressure, the difference between the actual pressure and the diamond cold-compression curve²⁵ at that density, with $\gamma = 1.15$ and $C_v = 2.0 \text{ J K}^{-1} \text{ g}^{-1}$ (ref. 26). This calculation assumes 100% conversion to diamond. These temperatures are sufficiently low relative to the melting temperature, and the time duration of the phase transformation sufficiently short (10 ns), that the rapid transition which we observe can be explained only by a martensitic transformation. □

Direct measurement of nitrogen gas fluxes from continental shelf sediments

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IT has been suggested that denitrification in continental shelf and slope sediments is the most important sink in the marine nitrogen cycle¹⁻⁴. This conclusion has been reached, not from direct measurements of denitrification in these areas, but rather from indirect estimates derived from pore-water models of diagenetic processes. In highly bioturbated continental shelf and slope sediments with steep pore-water gradients, such indirect estimates may not be applicable^{5,6}. I have now made direct, *in situ* measurements of denitrification in sediments of the eastern North Pacific continental margin by determining the flux of molecular nitrogen out of the sediments into the overlying water. Denitrification rates in continental shelf sediments measured in this fashion averaged $3.7 \text{ pmol N cm}^{-2} \text{ s}^{-1}$. The flux of nitrate from the overlying water into the sediments was only $1.5 \text{ pmol N cm}^{-2} \text{ s}^{-1}$, showing that most of the nitrogen gas production is coupled to nitrification within the sediments. The denitrification rates observed here are four to five times those estimated previously by indirect methods for these same sediments, and indicate the limitations of such indirect estimates. My results suggest that the global denitrification rate in shelf and slope sediments may be greater than previously thought, and confirm the importance of sedimentary denitrification in the marine nitrogen budget.

Denitrification is the process whereby combined nitrogen, principally NO_3^- , is used as the oxidant for organic matter and the nitrogen is reduced to N_2 (ref. 7). Denitrification takes place in the absence, or near absence of dissolved oxygen⁸ and is the major sink term in the marine budget of combined nitrogen. Inputs to the budget include atmospheric precipitation (40 Tg yr^{-1}), riverine input (25 Tg yr^{-1}) and *in situ* nitrogen fixation (25 Tg yr^{-1}). These inputs are balanced by losses due to permanent burial in marine sediments (20 Tg yr^{-1}), denitrification ($70\text{--}145 \text{ Tg yr}^{-1}$) and miscellaneous other processes (15 Tg yr^{-1})^{1,2}. Thus, depending on the true value of the

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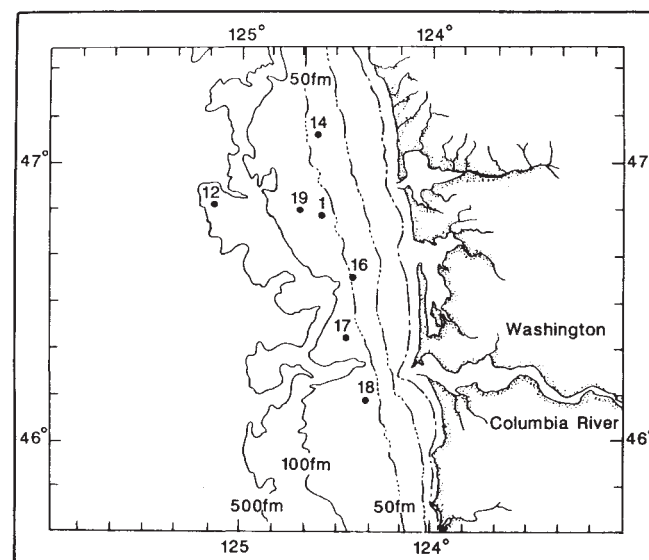


FIG. 1 Location of stations at which fluxes of N_2 and NO_3^- were measured.

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denitrification rate, the marine nitrogen budget may be balanced or unbalanced. There are two major sites of denitrification in the oceans: (1) the oxygen-deficient water column off western tropical coasts of Central America, South America and in the Indian ocean; (2) continental shelf and slope sediments. Although the water-column denitrification rate is relatively well constrained at about $60\text{--}80\text{ Tg yr}^{-1}$, estimates of sedimentary denitrification range from ~ 10 to 75 Tg yr^{-1} (refs 1, 7, 9).

The wide range in the estimates of the rate of sedimentary denitrification is due to differences in the individual, site-specific estimates from which it was extrapolated³. For the most part, the individual estimates are not based on direct rate measurements but on diagenetic models of pore-water concentrations of NO_3^- and NH_4^+ and models of benthic solute exchange^{3,4,10-12}. Given an input of organic matter sufficient to promote denitrification, several important factors help determine the overall rate: (1) the diffusion of NO_3^- across the sediment/water interface¹³; (2) the amount of NO_3^- , or other nitrogen oxides, supplied by nitrification (oxidation of NH_4^+) within the sediments^{10,11}; (3) the degree to which solute exchange between the sediments and overlying water is enhanced by macrobenthic activities (irrigation)¹⁴. In nearly all of the individual site-specific models one or more of these processes was not included (see Table 4, ref. 3). The goals of this work were to measure directly the denitrification rate by determination of the rate of N_2 increase in benthic flux chambers incubated *in situ*, thereby including all three important NO_3^- supplies, and, by comparing N_2 fluxes out of the sediments with NO_3^- fluxes into the sediments, to assess the quantitative importance of nitrification in supplying NO_3^- .

The measurements of benthic N_2 and NO_3^- fluxes were made at seven locations on the continental margin of Washington state in June and July 1988 (Fig. 1). Sedimentary organic carbon ranged from 0.7 wt% at station 18 to 1.6 wt% at station 1, which is typical for this area¹⁵. Because of the moderately intense oxygen minimum zone at mid-depth in the water column¹⁶ the concentration of dissolved oxygen in the water overlying the sediments decreased with increasing station depth (Table 1).

Nitrogen gas and NO_3^- fluxes were determined from changes in concentration with time in water overlying sediments enclosed by benthic flux chambers. Typical time series of concentration are shown in Fig. 2. In all cases N_2 concentrations increased with time (between 3% and 8%), indicating N_2 production within the sediments. In contrast, NO_3^- concentrations decreased with time. Table 1 lists fluxes calculated from the data. To facilitate comparison of different nitrogen species, fluxes are presented in units of pmol N ($1.0\text{ pmol N}_2 = 2.0\text{ pmol N}$ and

TABLE 1 Fluxes of N_2 and NO_3^- between sediments and overlying water

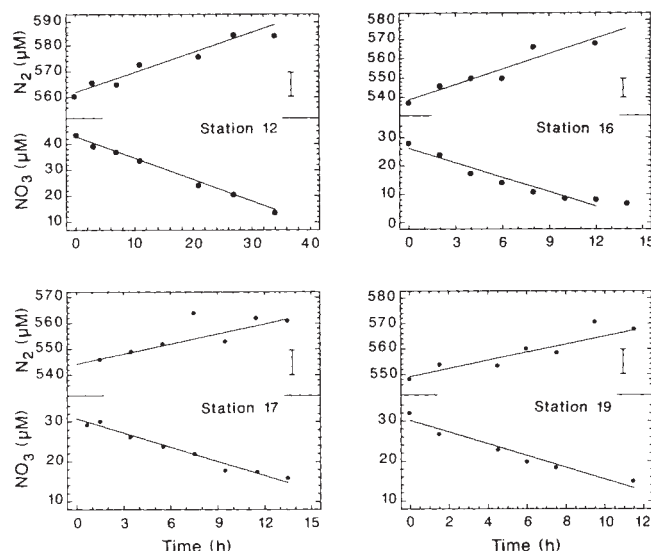
Station	Depth (m)	O_2 (μM)	NO_3^- (μM)	O_2 flux ($\text{pmol N cm}^{-2} \text{ s}^{-1}$)	NO_3^- flux ($\text{pmol N cm}^{-2} \text{ s}^{-1}$)	N_2 flux ($\text{pmol N cm}^{-2} \text{ s}^{-1}$)
1	115	110	30	12.3	1.4	-2.6
12	630	38	48	2.49	0.7	-0.9
14	114	127	29	21.2	1.9	-6.1
16	122	108	30	12.3	1.5	-3.6
17	120	112	31	16.5	1.9	-5.2
18	142	106	32	16.9	1.9	-4.0
19	98	—	—	11.0	1.4	-3.6

Negative values indicate a flux out of the sediments. Fluxes were calculated from the linear regressions shown in Fig. 2. Nitrite fluxes were also determined and, when measurable, were always out of the sediments. The magnitude of the nitrite flux was always $<10\%$ of the nitrate flux except at station 12, where it was 25%. Bottom water concentrations of O_2 and NO_3^- also given.

$1.0\text{ pmol NO}_3^- = 1.0\text{ pmol N}$). The nitrogen gas fluxes ranged from 0.9 to $6.1\text{ pmol N cm}^{-2} \text{ s}^{-1}$, with an average of $3.7\text{ pmol N cm}^{-2} \text{ s}^{-1}$. The lowest flux was from a relatively deep station on the continental slope, however, and for the other six stations the range was much narrower. Nitrate fluxes into the sediments ranged from 0.7 to $1.9\text{ pmol N cm}^{-2} \text{ s}^{-1}$ (average $1.5\text{ pmol N cm}^{-2} \text{ s}^{-1}$) and were always significantly lower than the corresponding N_2 fluxes. Again, the lowest flux was at the deepest station. For comparison, oxygen fluxes into the sediments, discussed in detail elsewhere¹⁷, are also given in Table 1. Oxygen fluxes varied by an order of magnitude, from 2.1 to $21.2\text{ pmol cm}^{-2} \text{ s}^{-1}$, with the lowest fluxes associated with overlying water having the lowest oxygen concentrations. The average O_2 flux was $13.2\text{ pmol cm}^{-2} \text{ s}^{-1}$.

Although other investigators have demonstrated N_2 production by sediments, frequently using ^{15}N tracers and metabolic inhibitors, these measurements were made in the laboratory on manipulated samples or sediment slurries^{18,21}, whereas I have measured N_2 production *in situ* on a typical open-ocean continental margin. Although the rates I obtained are comparable to values reported for estuaries and near coastal embayments ($1\text{--}7\text{ pmol N cm}^{-2} \text{ s}^{-1}$; ref. 22), they are significantly higher than previous estimates for the same area made by Christensen *et al.*¹². For August, the time most comparable to my June and July data, Christensen *et al.* estimate a denitrification rate of $0.8\text{ pmol N cm}^{-2} \text{ s}^{-1}$, much smaller than my estimate of $3.7\text{ pmol N cm}^{-2} \text{ s}^{-1}$. Their estimate was based on pore-water NO_3^- profiles of relatively coarse resolution ($\sim 1\text{ cm}$) and a model

FIG. 2 Typical time series of N_2 and NO_3^- concentration (μM) in the overlying water of the *in situ* flux chambers. Incubations were done using the benthic tripod described in ref. 5. The tripod was placed on the bottom, after which the flux chambers (410 cm^2) were partially implanted in the sediments and the lids closed entrapping 4–8 cm of overlying water. Samples for dissolved N_2 analysis were collected in 5-ml sample loops with water drawn through the loops by spring-actuated 60-ml syringes. While on the bottom, all sample operations were under the control of a pre-programmed microprocessor. On retrieval, water collected in the syringes was analysed for NO_3^- by Cd reduction²⁹. Dissolved N_2 was He-stripped from the samples and concentrations determined using a Varian gas chromatograph equipped with Carle thermal-conductivity detectors³⁰. The average N_2 increase was $23\text{ }\mu\text{M}$ (range $16\text{--}45\text{ }\mu\text{M}$) over a saturation value of $\sim 545\text{ }\mu\text{M}$. Tests revealed no N_2 contamination from any of the flux-chamber components. Precision of the N_2 analysis was determined in two ways. First, nine sample loops were filled from a single sample bottle and analysed. The average N_2 concentration of the nine samples was $550\text{ }\mu\text{M}$ with a standard deviation of $2.8\text{ }\mu\text{M}$. Second, during deployment no. 14, each time a sample was drawn within the flux chamber an ambient bottom-water sample was also taken. The seven ambient bottom-water samples averaged $547\text{ }\mu\text{M}$ with a standard deviation of $3.8\text{ }\mu\text{M}$. The error bars represent the 95% confidence interval for the ambient bottom-water samples.



of irrigation flux enhancement. A significant part of the difference between the two estimates is due to their lack of inclusion of the amount of denitrification that is coupled to nitrification within the sediments, which they suggest might contribute another $1.0 \text{ pmol N cm}^{-2} \text{ s}^{-1}$. This would bring their estimate to $1.8 \text{ pmol N cm}^{-2} \text{ s}^{-1}$, which is still smaller than my average value. The remainder of the difference is probably due to insufficient resolution of pore-water profiles, which are known to have very steep gradients in these sediments (refs 17, 23 and my unpublished data) and to shortcomings in the irrigation model. Nevertheless, the difference of a factor of four between these estimates points out the possible limitations of such indirect approaches.

The magnitude of the nitrogen flux (as N_2 gas) out of the sediments was about three times that of the flux (as NO_3^-) into the sediments (Table 1), indicating that only one-third of the overall denitrification could be supported by nitrate supplied from the overlying waters. The remaining NO_3^- was presumably supplied by nitrification of pore-water NH_4^+ produced during the oxidation of organic matter^{21,24,25}. Based on carbon oxidation rates estimated from rates of sulphate reduction and previous estimates of oxygen uptake, a sedimentary production rate of NH_4^+ of $\sim 2.9 \text{ pmol N cm}^{-2} \text{ s}^{-1}$ has been estimated for the Washington shelf¹². This is probably a lower limit, however, for the same reasons as discussed above for the discrepancy between the directly measured denitrification rates and the estimates. Even so, such a NH_4^+ production rate would be sufficient to account for the added N_2 flux. Others have examined the importance of nitrification coupled to denitrification through discrepancies in predicted and observed NH_4^+ fluxes^{4,6} or models^{10,14,24}, but in those studies the results could also be explained by oxidation of organic matter with a low N:C ratio, or unmeasured dissolved organic nitrogen fluxes, or shortcomings in model assumptions^{14,26,27}. The direct flux measurements clearly require nitrification within the sediments to supply the additional nitrogen oxides that must be denitrified to produce the observed N_2 flux.

The average denitrification rate of $3.7 \text{ pmol N cm}^{-2} \text{ s}^{-1}$ for the Washington continental margin is not only significantly greater than indirect estimates for the same sediments, it is also higher than most estimates for other continental shelf areas (see Table 4, ref. 3). It is, however, of similar magnitude to the estimate of $2.6 \text{ pmol N cm}^{-2} \text{ s}^{-1}$ derived for the Baltic sea²⁸. The data for the Washington continental margin derived from flux-chamber measurements should include all processes contributing to sediment denitrification (NO_3^- supplied from the overlying water, enhanced exchange due to irrigation and coupled nitrification and denitrification). The Baltic sea estimate was derived from a NO_3^- mass balance of the entire Baltic and also included all contributing processes. In contrast, estimates from most other continental margins are based on models of pore-water profiles, nearly all of which do not include one or more of the important processes. If the discrepancy between model-derived denitrification rates and the true rates for other shelf areas is as great as that on the Washington state shelf, then the global denitrification rate in marine sediments may be significantly underestimated. □

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Carbon–fluid equilibria and the oxidation state of the upper mantle

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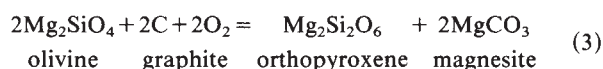
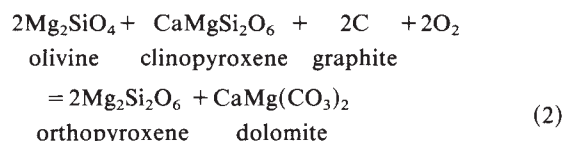
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It has been proposed that the oxidation state of the Earth's upper mantle is buffered by C–O fluids in equilibrium with elemental carbon^{1,2}. A large body of data on the oxygen fugacities (f_{O_2}) recorded by mantle rocks and their derivative melts now allows us to test this proposal. By comparing the measured f_{O_2} values with those calculated for carbon– CO_2 –CO–carbonate equilibria along appropriate mantle geotherms, we find the data to be wholly consistent with this hypothesis. Moreover, the calculated variation of f_{O_2} with temperature and pressure accounts for much of the observed correlation between the oxidation state of mantle samples and their tectonic provenance^{3,4}. The apparent buffering of mantle f_{O_2} requires modest quantities of mantle carbon and fluid, which do not exceed independently estimated values. The proposal is not compromised if parts of the mantle are fluid-undersaturated, or if the C–O fluid phase is diluted by other volatiles.

At relatively shallow levels in the mantle, equilibria between CO , CO_2 and carbon could buffer f_{O_2} through the equilibria¹ (termed CCO)



where carbon is present either as graphite or diamond. At higher pressures CO_2 and/or carbon should react with mantle silicates to produce carbonate, giving rise to the buffer equilibria^{5,6}



termed GEDOD and EMOG, respectively (the diamond equivalents of equilibria (2) and (3) are termed DEDOD and

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EMOD, respectively). An important feature of equilibria (1–3) is that, in contrast to silicate–oxide oxygen buffers, the values of f_{O_2} they define are extremely sensitive to both temperature and pressure⁷.

Estimates of mantle f_{O_2} are derived from thermodynamic calibrations of redox reactions between mantle minerals (olivine, pyroxene, spinel and garnet)^{3,4,8} or from experimental calibrations of ferric–ferrous equilibria in silicate melts⁹. These techniques are thought to be considerably more reliable than the intrinsic f_{O_2} measurements^{3,7,8} on which the original proposals of carbon–fluid buffering were based.

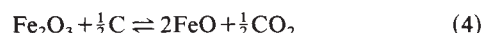
In Figure 1a we present data from spinel peridotite xenoliths in continental basalts from a wide range of continental volcanic provinces^{3,8}, including the southwestern United States, central Asia, the Massif Central and Japan. These data define temperature– f_{O_2} (T – f_{O_2}) arrays that lie close or parallel to the synthetic quartz–fayalite–magnetite (QFM) oxygen buffer at constant pressure (Fig. 1a). In general the subduction-related Japanese peridotites show slightly higher f_{O_2} than their continental counterparts, an apparently widespread feature of the mantle beneath island arcs^{3,4}. Each of the volcanic provinces sampled is characterized by a particular geothermal gradient in the underlying mantle. We can consider two extremes of likely continental geotherms: the first for stable continental craton¹⁰ and the second for the high-heat-flow Basin and Range province of the southwestern United States¹¹, whence many of the peridotite samples derive. From equilibria (1–3) we are able to calculate the variation in f_{O_2} that would be produced by C–O fluids in equilibrium with carbon along each geotherm. The calculated T – f_{O_2} curves for the two geotherms (Fig. 1b) bracket almost the entire observed variation in continental spinel peridotite xenoliths within the uncertainty of the measurements (± 0.5 log units⁸), notwithstanding the uncertainty in the heat-flow calculations used to construct the geotherms themselves¹¹. Preliminary data for high-pressure garnet peridotites¹² indicate log f_{O_2} between -0.2 and -4.0 at $1,450^\circ\text{C}$ and 65 kbar. Although these values are uncertain because of uncertainties in the models of garnet activity, at the reduced end of the range they lie close to our continental-shield geotherm (Fig. 1a) suggesting that carbon may control f_{O_2} well into the diamond stability field.

Data for the sub-oceanic mantle (Fig. 1b) derive from dredged oceanic peridotites¹³ and from mid-ocean ridge basalt (MORB) glasses⁹. Oceanic samples are reduced relative to continental peridotites, consistent with carbon–fluid control at the higher temperatures which prevail in the ocean basins. The most reduced samples in Fig. 1b derive from the hot-spot related peridotites from the Islas Orcadas¹³. Again, we have calculated carbon–fluid buffered f_{O_2} along two likely oceanic geotherms (Fig. 1b). The first corresponds to a mid-ocean ridge axis¹⁴; the second corresponds to 10-Myr-old oceanic lithosphere¹⁵, representing the likely maximum age for fracture-zone peridotites. The calculated T – f_{O_2} arrays encompass almost all of the oceanic data. The fit to the abyssal peridotites is especially good for data for which the temperature can be independently estimated. The inferred equilibration pressure for these peridotites is 10 ± 2 kbar, in good agreement with the suggestion that they represent fragments of the MORB source region¹³. The ridge-axis geotherm yields a range in f_{O_2} that is comparable to the spread observed in MORB glasses, when these data are extrapolated to pressure and temperature conditions appropriate to MORB genesis (Fig. 1b). Thus MORBs seem to be carbon-saturated in their source region and to record carbon–fluid buffered f_{O_2} corresponding to a range of depths between 10 and 60 km below the ridge crest. This is in accord with independent trace-element¹⁶ and petrological¹⁴ data on the overall depth range of MORB generation. Furthermore, the wide range of f_{O_2} observed in MORB glasses is incompatible with wholesale re-equilibration in sub-ridge magma chambers.

Figure 1 demonstrates that carbon–fluid buffering can account for the observed variation in upper mantle f_{O_2} . But for such a

proposal to be viable, we must demonstrate that there are sufficient C–O fluids and elemental carbon in the mantle to provide the requisite buffer capacity and that the model remains valid for fluid-undersaturated parts of the mantle or when the C–O fluid phase is diluted by other volatile species such as H_2O .

The powerful buffering capacity of small amounts of elemental carbon is apparent from the phenomenon of auto-reduction during measurements of intrinsic f_{O_2} ^{7,17}. The principal redox reaction in the mantle can be written



In the minerals used to monitor mantle f_{O_2} , Fe_2O_3 is lodged primarily in the spinel and garnet phases, which make up on average only 3 wt% of a typical mantle lherzolite. The range of Fe_2O_3 content between the most reduced and most oxidized mantle spinels, a range of over 4 log units in f_{O_2} , is only 1–6 wt%^{8,13}, corresponding to a net increase on oxidation of 10^{-5} moles of Fe_2O_3 per gram of mantle rock. The reverse is true for reduction. Thus to effect the maximum observed change in mantle f_{O_2} requires only 5×10^{-6} moles of carbon, carbonate or CO_2 per gram of mantle. In terms of solid phases, this is only 60 p.p.m. elemental carbon or 420 p.p.m. MgCO_3 . In terms of CO_2 , 200 p.p.m. by weight fluid or volumetric fluid:rock ratios (at 15 kbar and $1,100^\circ\text{C}$) of 1:1,600 are required. Sato and Valenza¹⁸ use similar mass-balance calculations to show that the presence of < 500 p.p.m. carbon can account for the observed variation in f_{O_2} in the Skaergaard ultramafic intrusion. Our estimates are consistent with independent evidence on the carbon and fluid content of the mantle. For example, Marty and Jambon¹⁹ propose a mean mantle carbon content of 1,000 p.p.m. based on mantle C/ ^3He ratios, and Turner *et al.*²⁰ used $^{40}\text{Ar}/\text{Cl}$ ratios of fluid inclusions in diamonds to estimate that H_2O – CO_2 fluids are present at levels up to 100–200 p.p.m. by weight in the mantle. That the mantle contains elemental carbon is supported by several lines of evidence⁷, including the existence of diamonds beneath ancient cratons, the presence of graphitic inclusions in ilmenite megacrysts from kimberlites²¹, and the occurrence of trace amounts of graphite in many mantle-derived magmatic rocks^{18,22}.

Further constraints on the mantle CO_2 budget can be provided by MORB. Using the primary bubble content of fresh MORB, Bottinga and Javoy²³ estimate a pre-degassing CO_2 content of 0.68 wt%. If we make the simplifying assumption that MORB represents 10% partial melt of mantle this value corresponds to ≥ 680 p.p.m. CO_2 in the source region. This value is in the range given above and supports the hypothesis that there is sufficient carbon and C–O fluid in some parts of the mantle to control f_{O_2} .

Although the nature and distribution of mantle volatiles is a contentious issue, it seems unlikely that the mantle will contain a ubiquitous binary C–O fluid. The common occurrence of hydrous phases, such as amphibole or phlogopite, in mantle peridotites testifies to the presence of at least some H_2O , and the assumption of CO_2 saturation in the MORB source region²⁴ is by no means universally upheld. As equilibria (1a) and (1b) apply only to pure C–O fluids, we must assess what impact fluid-undersaturation or dilution by H_2O would have on f_{O_2} control.

Where C–O fluids are diluted by other volatiles or wholly dissolved in a basaltic partial melt, equilibria (1a) and (1b) acquire another degree of freedom and cannot in themselves control f_{O_2} without the imposition of a further constraint⁶. In the carbonate-bearing mantle, however, equilibria (2) and (3) buffer both the activity of fluid species and f_{O_2} , although these equilibria do not require the presence of a free fluid phase⁶. In the absence of a pure C–O fluid, the f_{O_2} defined by equilibria (1a) and (1b) is determined by the activity of CO_2 (a_{CO_2}) in the fluid or melt phase. Under high-temperature low-pressure conditions a_{CO_2} is also important. As log a_{CO_2} and log f_{O_2} are linearly dependent, a value of $a_{\text{CO}_2} = 0.1$ in the fluid or melt will reduce f_{O_2} by only one log unit relative to CCO at the same

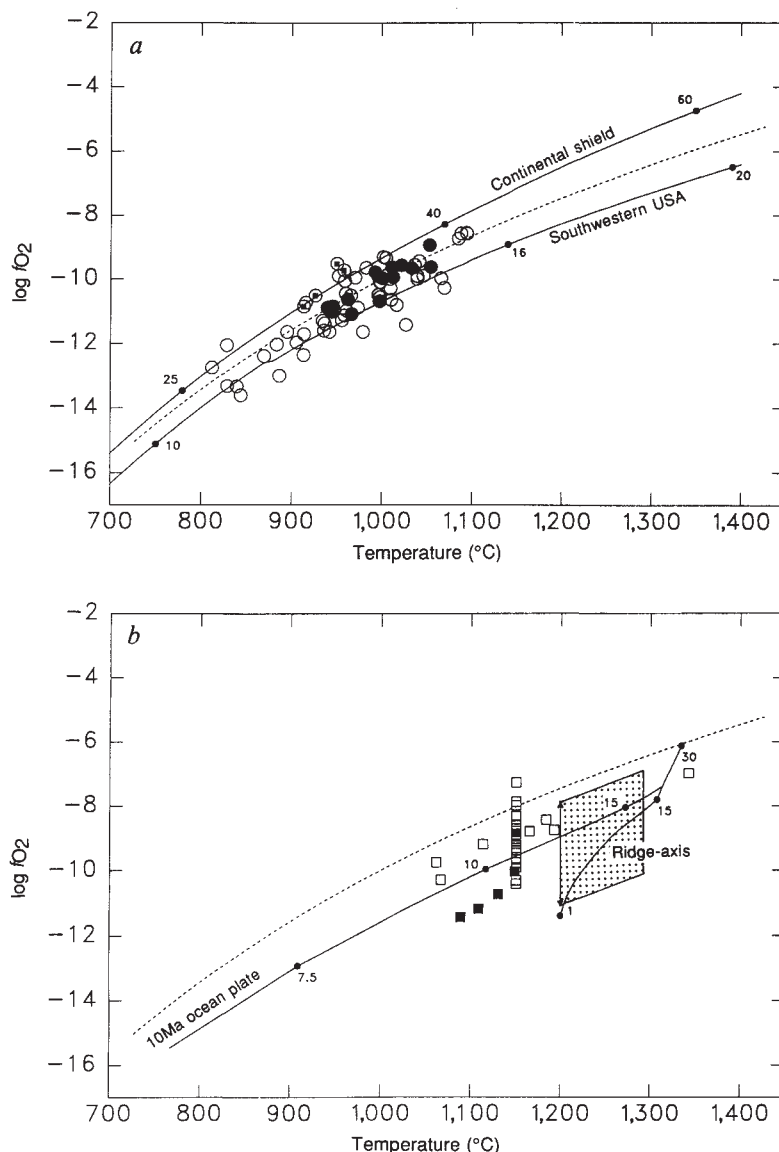
pressure and temperature⁶. If a_{CO_2} is maintained at or above this value in CO_2 -undersaturated mantle melts or in H-C-O mantle fluids then there will be no significant deviation from CCO. In the case of H-C-O fluids this assertion is best illustrated by simple equation-of-state calculations²⁵, which show that the H_2O content of H-C-O fluids in equilibrium with elemental carbon increases to a maximum molar ratio $\text{H}_2\text{O}/(\text{H}_2\text{O} + \text{CO}_2) = 0.9$ at ~ 0.8 log units below CCO²⁶. Thus, given carbon-saturation, even relatively water-rich H-C-O fluids will impose a f_{O_2} close to that defined by the CCO buffer. In the carbonate-free parts of the mantle the $\text{H}_2\text{O}/(\text{H}_2\text{O} + \text{CO}_2)$ ratios are probably controlled either by hydrous mantle minerals, such as amphibole²⁷, beneath the solidus or by silicate melts above the solidus. As both phases show a strong preference for H_2O at these pressures²⁸, it is likely that the $\text{H}_2\text{O}/(\text{H}_2\text{O} + \text{CO}_2)$ ratio of the upper mantle is maintained at sufficiently low values to preclude appreciable deviation from CCO.

For the case of a fluid-undersaturated mantle we refer to the data of Pan *et al.*²⁹ on CO_2 solubility in basalt. They show that Bottinga and Javoy's estimate of 0.68 wt% CO_2 in MORB²³ can be reconciled with CO_2 -saturated MORB genesis only at pressures ≤ 9 kbar. At higher pressures the partial melts generated will be CO_2 -undersaturated such that all CO_2 is dissolved in the melt phase and the position of equilibria (1a) and (1b) will be determined by $a_{\text{CO}_2}^{\text{melt}}$. Deviation of this value from unity will reduce f_{O_2} relative to CCO. The data of Pan *et al.* suggest,

however, that large reductions in f_{O_2} are unlikely. The solubility of CO_2 at $\sim 1,500^\circ\text{C}$ and 20 kbar is 1.45 wt% CO_2 (ref. 29). Thus, assuming a linear relationship between CO_2 weight fraction and activity at these low concentrations, it seems unlikely that the $a_{\text{CO}_2}^{\text{melt}}$ in a melt with 0.68 wt% CO_2 will drop much below 0.5 even for these higher-pressure conditions of MORB genesis. The corresponding reduction in f_{O_2} is only 0.3 log units, that is, within the uncertainty of the measurements. The position of the CCO buffer is therefore insensitive to the absence of a pure C-O fluid provided that a CO_2 -bearing melt phase is present. The high mobility of small melt fractions in the mantle³⁰ suggests that CO_2 -undersaturated melts are potentially just as efficient at controlling f_{O_2} as pervasive C-O or H-C-O fluids. Carbonatitic partial melts may have a similar role in the deeper carbonate-bearing parts of the mantle²⁸.

Our calculations suggest that even in the absence of pure C-O fluids the f_{O_2} of a carbon-saturated mantle will lie close to CCO. Equilibria (1a) and (1b) seem to be sufficiently robust to preclude any significant deviation from CCO except where the mantle carbon budget is perturbed to the extent that elemental carbon is no longer a stable phase. Situations in which this might occur include carbon consumption during extensive partial melting, the recycling of relatively oxidized lithosphere into the mantle, and the fluxing of substantial volumes of hydrous fluids into the mantle. Conversely, any flux of carbon-bearing fluids from the deeper parts of the mantle²⁶ will serve to replenish

FIG. 1 Temperature variation of f_{O_2} in mantle-derived rocks. a, Peridotite xenoliths from continental basalts (data from refs 3, 8, and unpublished data of B.J.W. and D. Ionov) at an estimated pressure of 15 kbar. ●, Samples from San Carlos and Kilbourne Hole in southwestern United States; ○, samples from Japan; ○, samples from Massif Central and central Asia. The solid curves show calculated f_{O_2} variation as buffered by carbon-fluid equilibria (1-3) along representative geotherms for old continental shield¹⁰ and the relatively hot southwestern United States¹¹. Numbers adjacent to the curves denote pressure in kbar. The f_{O_2} along the continental geotherm is controlled by EMOG at temperatures $< 1,140^\circ\text{C}$ and by EMOD at higher temperatures. Along the southwestern-US geotherm f_{O_2} is controlled by CCO ($< 880^\circ\text{C}$), GEDOG ($< 1,100^\circ\text{C}$) and EMOG/EMOD at higher temperatures. The broken line is the QFM reference buffer at 15 kbar. b, Oceanic mantle samples. □, Dredged abyssal peridotites¹³ at an estimated pressure of 10 kbar. Where no geothermometry is available temperature is assumed to be $1,150^\circ\text{C}$ (ref. 13). ■, Hot-spot-related peridotites from Islas Orcadas. The arrow denotes the range of MORB glasses at 1 bar and an arbitrary temperature of $1,200^\circ\text{C}$ (ref. 9). The adjacent stippled area denotes extrapolation of the MORB glass data to 15 kbar and $1,300^\circ\text{C}$ (ref. 34), conditions appropriate for MORB generation. The solid curves show calculated f_{O_2} as buffered by CCO (equilibria 1a and 1b) along geotherms of a ridge-axis¹⁴ and 10-Myr-old oceanic crust¹⁵. The dashed line is QFM at 15 kbar. All spinel peridotite data have been recalculated according to the Wood-Nell spinel-olivine-orthopyroxene oxygeobarometer (given in the appendix to ref. 3). Values of f_{O_2} calculated in this way use estimated pressures owing to the pressure insensitivity of the oxygeobarometer (~ 0.03 log units per kbar) and the lack of suitable geobarometers for spinel peridotites. CCO buffer curves have been calculated for pure C-O systems using data from refs. 25, 35 and 36. The curves will be lowered by up to 0.8 log units in f_{O_2} if H_2O is present in the fluid at molar ratios $\text{H}_2\text{O}/(\text{H}_2\text{O} + \text{CO}_2) \leq 0.9$ and by ≤ 0.3 log units if $a_{\text{CO}_2}^{\text{melt}} \geq 0.5$ for fluid-undersaturated mantle.



upper-mantle carbon supplies. The first possibility is difficult to quantify, but can be visualized conceptually. During the early stages of mantle melting the ferric-ferrous ratios of the melt and residual solid will be determined by values of f_{O_2} at or close to CCO. If carbon is consumed during melting the ferric-ferrous ratio of the melt will decrease unchecked until the melt segregates from its source. The extent to which carbon-absent melting will proceed is unknown, although the large variation of f_{O_2} in MORB glasses (Fig. 1b) may reflect this process as well as the aggregation of polybaric mantle melts. The second possibility is addressed by Wood *et al.*³ with reference to the source of island-arc basalts. The third possibility may also be appropriate to subduction zones where appreciable H_2O is introduced into the mantle by the subducting slab³¹. If the $H_2O/(H_2O+CO_2)$ ratio in the hybrid mantle fluids exceeds ~ 0.9 , any mantle carbon

will be completely oxidized by H_2O (ref. 26), enabling f_{O_2} to rise unbuffered above CCO. We propose that recycling of hydrous fluids in subduction zones may account for the relatively oxidizing nature of subduction-related peridotites^{3,4} (Fig. 1a) and the more oxidized nature of arc magmas in general³².

Carbonate-bearing equilibria (2) and (3) may control f_{O_2} in the deeper parts of the mantle⁵, for example, at pressures >12 kbar along the geotherm of the southwestern United States (Fig. 1a). Although carbonate is rare in mantle xenoliths, including those in Fig. 1a, it has recently been shown experimentally³³ that carbonates in mantle assemblages rapidly decompose on decompression. Thus the apparent lack of carbonate in mantle samples in the quantities required to control f_{O_2} (~ 0.05 vol% $MgCO_3$) may be entirely an artefact of the volcanic processes by which they are brought to the surface. □

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Owenetta and the origin of turtles

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THE origin and relationships of turtles have fascinated and puzzled generations of palaeontologists. Among living amniotes only turtles, crocodiles and mammals have substantial fossil records, extending into the Triassic (200 Myr). These vertebrates have attracted much attention and the broader aspects of crocodilian and mammalian evolutionary relationships are relatively well known. Therefore, it is surprising that the origins and relationships of the Testudines have remained unresolved. Numerous groups of extinct tetrapods^{1–7} have been cited as possible turtle relatives, including the Captorhinidae^{8–12}. New specimens of the small reptile *Owenetta* from the Upper Permian and Lower Triassic sediments of South Africa provide strong evidence that a group of primitive amniotes, the procolophonids, are the closest sister-group of turtles.

Procolophonid reptiles are small to medium-sized primitive amniotes that first appear in late Palaeozoic (Upper Permian) deposits of South Africa. The fossil record of procolophonids extends to the end of the Triassic (early Mesozoic), and indicates that this was a diverse and successful group with worldwide distribution. Procolophonids are the last known members of the initial adaptive radiation of amniotes, and were probably ecological analogues to some of the small and medium-sized diapsid reptiles that eventually replaced them. Procolophonids are characterized by the following osteological features: the orbital shelf of the prefrontal has a medial process, the ventral edge of

the skull is concave in the region of the jugal and the quadratojugal, the ectopterygoid is reduced in size and lacks teeth, and despite their relatively small size, procolophonids have three sacral vertebrae. The early procolophonids have simple peg-like teeth, whereas the advanced Triassic forms develop bulbous, transversely expanded teeth. The larger Triassic procolophonids develop posterolateral horn-like projections on the quadratojugal.

The oldest known procolophonid is *Owenetta*^{13,14}, a small insectivorous reptile recovered from Upper Permian sediments of the Karroo, South Africa. The study of recently discovered skulls and articulated skeletons (Fig. 1) of this poorly known, primitive procolophonid has led to the discovery of numerous shared derived osteological features (synapomorphies) between procolophonids and turtles. Comparisons between procolophonids such as *Owenetta* and turtles have also been facilitated by a recent thorough redescription of the oldest known turtle, *Proganochelys*¹² (Fig. 2), and the discovery of the Early Jurassic turtle, *Kayentachelys*¹⁵. Detailed new information about the cranial anatomy of these forms is particularly important because the great specialization of the postcranial skeleton of all turtles (associated with the development of the shell) appears to have obscured most evidence (synapomorphies) of testudine origins and relationships.

Procolophonids and testudines form a monophyletic group that excludes all other amniotes because they share the following derived features which appear in the primitive condition in all other Palaeozoic reptiles: (1) the cultriform process is greatly reduced in length; (2) the teeth on the transverse flange of the pterygoid are lost, and are replaced by a ventral ridge; (3) a distinctly shaped anterodorsal expansion of the maxilla is formed directly posterior to the external naris; (4) the prefrontal and palatine are massively buttressed against each other; (5) the dorsal process of the quadrate is exposed laterally, but the

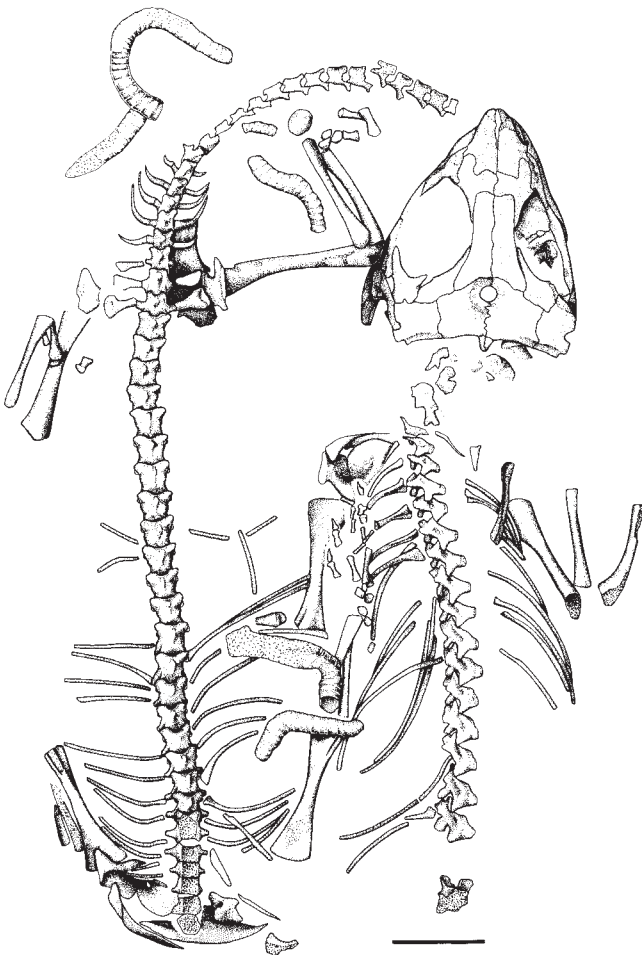
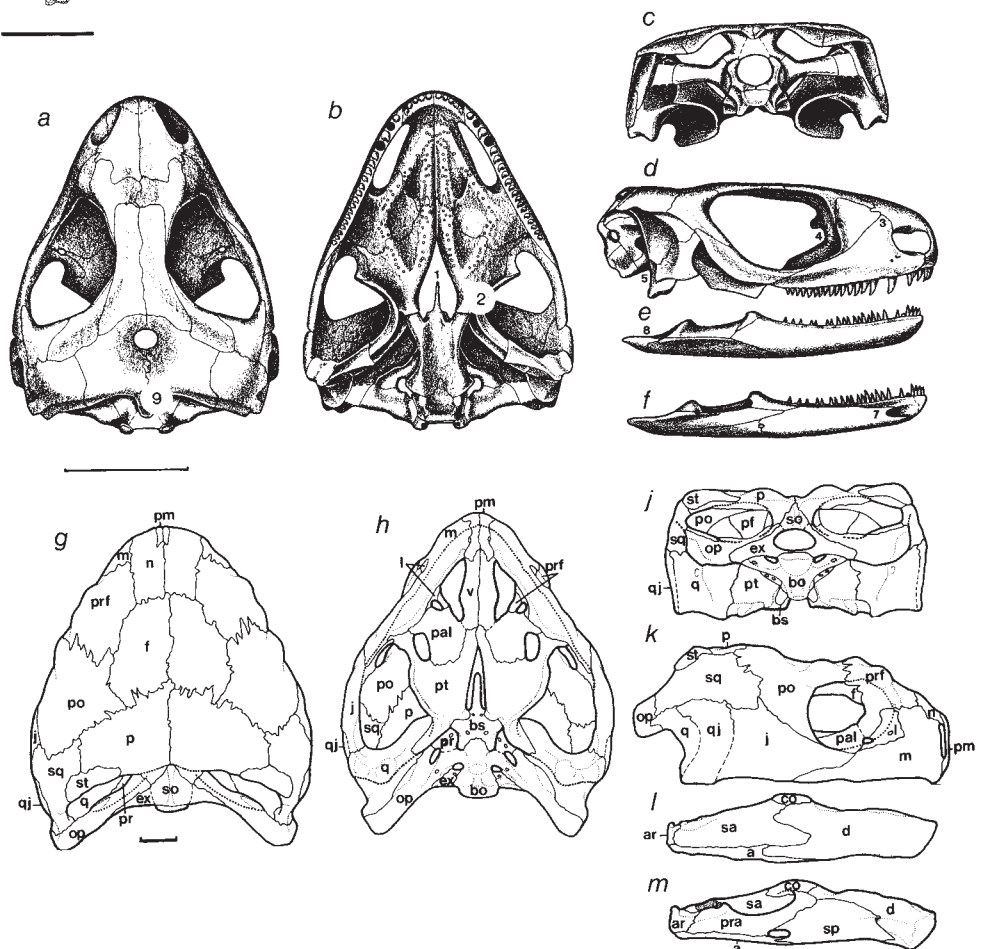


FIG. 1 Two articulated skeletons of the primitive procolophonid *Owenetta* from Tweefontein, Bethulie, South Africa (Cistecephalus zone, Beaufort Series, Lower Triassic). The specimen (BPI 4195) is housed at the Bernard Price Institute of Paleontology, University of Witwatersrand, Johannesburg, South Africa. The neck and head of one skeleton and the pelvis and tail of the other were destroyed by surface erosion before the discovery of this specimen. Scale bar, 1 cm. Small, millepede-like arthropods have been found in abundance in the rib cage and near the skeletons, indicating that these small scavenging arthropods were probably feeding on the two carcasses before burial and fossilization. *Owenetta* has been included in the Family Procolophonidae by all previous palaeontologists who studied this poorly known reptile, including Gow¹⁴, who describes some of the features that we discuss, but did not realize that they were synapomorphies with testudines. We do not include the enigmatic nyctophretids from the late Permian of the USSR within the Procolophonidae.

FIG. 2 Reconstructions of *Owenetta* (a–f) and *Proganochelys* (g–m) skulls in dorsal (a, g), ventral (b, h), occipital (c, i), and lateral (d, k) views, and mandible in lateral (e, l) and medial (f, m) views. *Owenetta*, originally described on the basis of a partial skull from New Bethesda, Graaff Reinet, South Africa (Daptocephalus zone, Beaufort Series, Upper Permian), is now represented by numerous skulls from the same region. The reconstructions are based on specimen shown in Fig. 1. *Proganochelys* is the oldest known turtle, from the late Triassic of Germany. The outline skull reconstructions of *Proganochelys* are modified slightly from that of Gaffney¹². Abbreviations: a, angular; ar, articular; bo, basioccipital; bs, basisphenoid; co, coronoid; d, dentary; ex, exoccipital; f, frontal; jugal; l, lacrimal; m, maxilla; n, nasal; op, opisthotic; p, parietal; pal, palatine; pf, postfrontal; pm, premaxilla; po, postorbital; pr, prootic; pra, prearticular; prf, prefrontal; pt, pterygoid; q, quadrate; qj, quadratojugal; sa, surangular; so, supraoccipital; sp, splenial; sq, squamosal; st, supratemporal; v, vomer. The numbers refer to the synapomorphies discussed in the text.



edge of the well developed tympanic notch is formed by the squamosal and the enlarged quadratojugal (also present in *Proganochelys* (personal observation), although not possible to show in Fig. 2k); (6) the slender stapes has lost both its dorsal process and foramen (not shown in the figures); (7) on the lower jaw the distinctly shaped anterior edge of the splenial is excluded from the symphysis; (8) the dorsal surface of the retroarticular process is broad and concave, and is formed by at least three bones, the articular, angular and prearticular; (9) the postparietal is greatly reduced or lost (see Fig. 2 for illustration of these points); (10) the entepicondylar foramen of the humerus is lost. No other group of Palaeozoic reptiles has these derived features. Although some Palaeozoic reptiles¹⁶ appear to possess a tympanic notch, the configuration of the surrounding elements is entirely different from those of procolophonids and turtles. Advanced diapsids also reduce and eliminate the postparietal, but the most primitive diapsids retain the primitive condition of well developed postparietals¹⁷. Even though only one postcranial synapomorphy (the loss of the entepicondylar foramen) can be recognized, this derived condition is not seen in any other Palaeozoic reptile.

Such a strong case has never been made to demonstrate close relationships between turtles and any other group of primitive tetrapods. By contrast, the most recent widely accepted hypothesis of close captorhinid-turtle relationships^{10,12} is supported by only four synapomorphies. Two of these characters, however, the absence of the tabular and the presence of the foramen orbito-nasale, are not unique to turtles and captorhinids, but are also found in other reptiles, including procolophonids.

In addition to solving a problem that has puzzled generations of palaeontologists, our results could alter considerably our concepts of turtle evolution. The use of procolophonids (instead of captorhinids) as the outgroup of turtles requires changes to the diagnosis of the Testudines: both the presence of a solid, rod-like imperforate stapes and the distinctive morphology of the quadrate-squamosal-quadratojugal complex (probably associated with the development of a tympanum and an impedance-matching middle ear) have been used to diagnose turtles¹⁰, but are also present in procolophonids. These new results also alter significantly our interpretation of the testudine fossil record. If captorhinids are the closest sister-group of turtles, then the Testudines must have arisen at least as early as the early Permian, and possibly the late Carboniferous. As the oldest known turtle comes from Upper Triassic strata, this implies an unusually long gap in the fossil record (more than 100 Myr), longer than any other group of modern reptiles. If, however, Testudines is the sister-group of procolophonids, then this group of extant reptiles may have originated as late as the late Permian. This second hypothesis implies a much shorter gap and hence the fossil record associated with the origin of turtles may not be as incomplete as previously thought. □

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Endogenous nitric oxide release required for long-term synaptic depression in the cerebellum

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CONJUNCTIVE stimulation of climbing and parallel fibres in the cerebellum evokes a long-term depression of parallel-fibre Purkinje-cell transmission^{1,2}, a phenomenon implicated as the cellular mechanism for cerebellar motor learning³. It is suspected that the increase in cyclic GMP concentration that occurs after activation of climbing fibres⁴ is required to evoke long-term depression³. Excitatory amino acids are known to cause the release of nitric oxide (NO), resulting in elevation of the cGMP level in the cerebellum⁵. Here we report that endogenous NO is released after stimulation of climbing fibres, that long-term depression evoked by conjunctive stimulation of parallel and climbing fibres is blocked by haemoglobin (which strongly binds NO) or L-N^G-monomethyl-arginine (an inhibitor of NO synthase), and that exogenous NO or cGMP can substitute for the stimulation of climbing fibres to cause long-term depression in rat cerebellar slices. These results demonstrate that the release of endogenous NO is essential for the induction of synaptic plasticity in the cerebellum.

Release of NO was recorded by electrochemical NO probes⁶ in the molecular layer of cerebellar slices. Stimulation of white matter (20 Hz, 5 s) evoked increases in NO concentration of between 20 and 75 (47 ± 3, ± s.e.m., *n* = 20) nM. The endogenous NO response showed a similar dependence on the cathode voltage of the NO probe as did probe currents induced by a standard NO solution (100 nM; Fig. 1a, b). Haemoglobin (bovine, 10 μM), which strongly binds NO (refs 5, 6), attenuated the NO response by 46–58% (*n* = 3; Fig. 1c). L-N^G-monomethyl-arginine (NMMA, 100 μM), an inhibitor of NO synthase (ref. 7), blocked the NO response by 82–87% (*n* = 3). This effect was antagonized by L-arginine (Arg, 1 mM; Fig. 1d).

Release of NO was also recorded in slices obtained from rats treated with 3-acetylpyridine (3AP), which selectively produces lesions in the inferior olive and removes climbing fibres⁸ (Fig. 1e). The estimated NO release was between 5 and 13 (9 ± 2, *n* = 5) nM, and was significantly less than that in normal slices (*P* < 0.001, Mann-Whitney U-test). A low-Ca²⁺ (0.24 mM) medium blocked the NO release almost completely and reversibly in normal slices (Fig. 1f). Among various glutamate blockers, L-glutamic acid diethyl ester (GDEE, 10 mM) suppressed the NO response by 61–66% (*n* = 3; Fig. 1g), while kynurenate (10 mM), 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX, 10 μM) and D-2-amino-5-phosphonovaleric acid (APV, 100 μM) showed no clear effect (*n* = 3 for each). These results suggest that release of endogenous NO is triggered by a transmitter released from climbing fibres, which may activate unknown receptors.

Long-term depression can be monitored as changes in the extracellular potassium concentration ([K⁺]_o) after stimulation of parallel fibres in the molecular layer⁹. Conjunctive stimulation of parallel fibres and the white matter evoked long-term depression of the K⁺ response (Fig. 2a, b). Depression was also tested in slices treated with 3AP (Fig. 2c), and found to be

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TABLE 1 Amplitude of long-term depression

	30 min*	60 min
conjunctive stimulation	34 ± 9%†	42 ± 10
in 3AP slices	1 ± 6 (P < 0.005)‡	-4 ± 10 (P < 0.005)
in Hb medium	7 ± 7 (P < 0.01)	-1 ± 15 (P < 0.005)
in NMMA medium	0 ± 5 (P < 0.005)	-8 ± 6 (P < 0.005)
	30 min	60 min
SNP + PFS	51 ± 9	66 ± 9
SNP without PFS	12 ± 4 (P < 0.005)	25 ± 8 (P < 0.01)
SNP in 3AP slices	44 ± 14	54 ± 11
	40 min	60 min
Br-cGMP + PFS	23 ± 8	32 ± 9
Br-cGMP without PFS	-4 ± 7 (P < 0.02)	-3 ± 6 (P < 0.005)

3AP, 3-acetylpyridine; Hb, bovine haemoglobin; NMMA, L-N^G-monomethyl arginine; SNP, sodium nitroprusside; PFS, parallel-fibre stimulation; Br-cGMP, 8-bromo cGMP.

* Time after cessation of conjunctive stimulation, SNP or Br-cGMP application.

† Mean ± s.e.m., *n* = 5 for each group.

‡ Mann-Whitney *U*-test.

but SNP alone caused only a slight depression (Fig. 3*a, b*; Table 1). In slices treated with 3AP, SNP with parallel-fibre stimulation evoked long-term depression not significantly different from that seen in normal slices (Fig. 3*c*; Table 1).

The K⁺ response is only partially blocked by kynurenate, and the kynurenate-resistant K⁺ response is probably attributed to presynaptic activities in parallel fibres⁹. The remaining K⁺ response during kynurenate application was not markedly affected by SNP plus parallel-fibre stimulation (Fig. 3*d*). Therefore long-term depression seems specific to parallel-fibre/Purkinje-cell transmission.

A target of NO is a soluble guanylate cyclase⁵. To test whether climbing-fibre stimulation or NO can be replaced by exogenous cGMP, 8-bromo-cyclic GMP (Br-cGMP, 1 mM), a membrane-permeable derivative of cGMP, was applied to slices for 40 min. Long-term depression was evoked by Br-cGMP plus parallel-fibre stimulation, but Br-cGMP application alone was not so effective (Table 1).

Stimulation of white matter evoked marked depression and NO release in normal slices but not in slices from 3AP-treated rats, which showed clear and typical disturbance in motor behaviour. The effects of 3AP on the cerebellum seemed specific to climbing-fibre destruction, because no change was seen in the K⁺ response or in long-term depression evoked by SNP plus parallel-fibre stimulation in 3AP slices. Furthermore, climbing-fibre activation causes elevation of cGMP (ref. 4), which probably mediates the effects of NO. We conclude that the effects of white-matter stimulation are attributed to climbing-fibre activation.

A calmodulin-dependent NO synthase has been isolated from the cerebellum¹⁰, and intracellular injection of EGTA into Purkinje cells has been shown to block long-term depression¹¹. Therefore endogenous NO release might be triggered by increases in intracellular calcium in Purkinje-cell dendrites following climbing-fibre activation and the resultant activation of an NO synthase. But two lines of evidence oppose this view.

significantly lower than in normal slices (Table 1). To study the role of endogenous NO release in long-term depression, haemoglobin (1 μM) was applied to slices (Fig. 2*d*, and Table 1). Although no marked effect on the K⁺ response was observed, depression was blocked almost completely and was significantly less than that in normal medium. NMMA (100 μM) also blocked long-term depression (Table 1).

To test whether endogenous NO can be replaced with exogenous NO, the K⁺ response to parallel-fibre stimulation was evoked in a medium containing sodium nitroprusside (SNP, 3 mM), which releases NO (refs 5, 6). Marked long-term depression was evoked after SNP plus parallel-fibre stimulation,

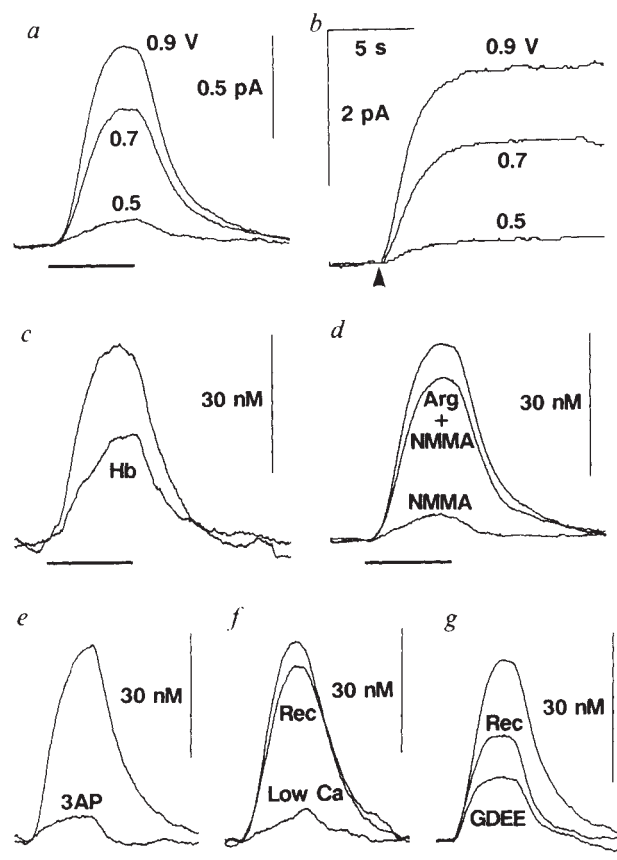


FIG. 1 Endogenous NO release in the molecular layer of the rat cerebellar slices. *a*, Currents in an NO probe inserted into the molecular layer during white-matter stimulation. Horizontal bar, white-matter stimulation at 20 Hz for 5 s. Each of the superimposed traces was recorded at a different cathode voltage in the NO probe, as shown. *b*, Currents induced by a 100 nM step increase in NO concentration at the arrow head. Data in *a* and *b* were recorded by the same NO probe. *c*, Superimposed NO responses evoked by white-matter stimulation in normal and 10 μM haemoglobin (Hb, bovine)-containing media. *d*, NO release responses in normal, NMMA (100 μM) and NMMA plus L-Arginine (Arg, 1 mM) media. *e*, Typical endogenous NO release in normal and 3AP slices. *f*, NO release in a normal slice before, during and after (recovery, Rec) application of a low-Ca²⁺ (0.24 mM) medium. *g*, NO release before, during and after (Rec) application of 10 mM GDEE. In *a* and *c-g*, each trace represents the averaged result of five responses recorded at 2-min intervals.

METHODS. The NO probe has been described elsewhere⁶. It was made from a small glass pipette filled with 30 mM NaCl and 0.3 mM HCl. After the opening of the pipette was sealed with a thin rubber membrane, the cathode and anode were inserted into the pipette, and connected to a current-voltage converter. The cathode was located as close as possible to the membrane at the opening. NO gas diffused into the pipette through the rubber membrane is oxidized at the surface of the cathode kept at +0.9 V, and is detected as the current passing through the cathode. This current is linearly correlated with NO concentration at the tip⁶. Parasagittal slices (thickness, 400 μm) were prepared from the vermis of male Wistar rats (250–350 g) with a vibrating slicer and kept in a warmed (35°C) and oxygenated medium⁶. An NO probe was inserted into the molecular layer. The white matter was stimulated with biphasic pulse-trains (intensity, ±600 μA; duration of each biphasic pulse, 2 ms; frequency, 20 Hz; duration of pulse-train, 5 s). To make selective climbing-fibre lesions, 3-acetylpyridine (75 mg per kg), harmaline (15 mg per kg) and niacinamide (300 mg per kg) were injected intraperitoneally into rats at 3 and 1.5 h intervals⁸, respectively. Rats were used for recordings between 11 and 40 days after the injection.

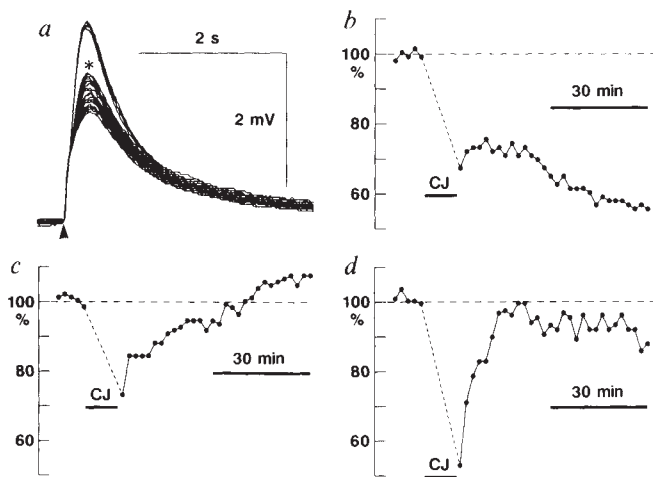


FIG. 2 The $[K^+]_o$ increase response evoked by parallel-fibre stimulation (PFS), and its long-term depression. The basic methods have been described elsewhere⁹. *a*, Recorded voltage changes of a K^+ -sensitive microelectrode placed in the molecular layer of slices. Arrow head, PFS. Responses to PFS were recorded before and after (asterisk) conjunctive stimulation of parallel fibres and the white matter, and were superposed. Traces were recorded at 2-min intervals. *b*, Changes in maximal amplitude of the K^+ response before and after the conjunctive stimulation (horizontal bar, CJ) of the parallel fibre and the white matter. The ordinate is the relative amplitude normalized by the amplitude of three successive K^+ responses immediately before CJ. Data are the same as those shown in *a*. *c*, Effects of CJ on the K^+ response recorded in a 3AP slice. *d*, Effects of CJ on the K^+ response recorded in a normal slice during application of Hb (1 μ M) throughout the recording. METHODS. Parallel fibres were stimulated with five successive pulses (duration of each pulse, 300 μ s; interval between pulses, 5 ms). The intensity was adjusted so that the maximal amplitude of the K^+ response was a few millivolts. If the required current intensity exceeded 70 μ A, the slice was discarded. The white matter was also stimulated with the same time-parameters, but the intensity was fixed to 800 μ A. For conjunctive stimulation, 60 pairs of parallel-fibre and white-matter stimulation were applied at 10-s intervals. Parallel-fibre stimulation preceded white-matter stimulation by 200 ms in each pair.

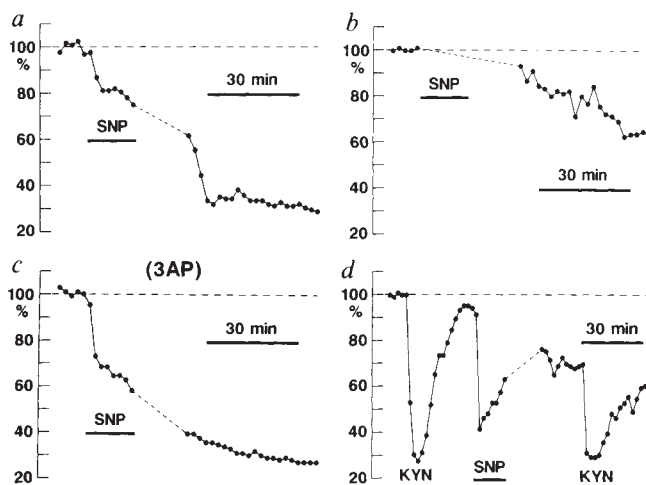


FIG. 3 Long-term depression of the K^+ response during sodium nitroprusside (SNP) application. *a*, Long-term depression evoked by a combination of parallel-fibre stimulation (PFS) and SNP (3 mM) application for 16 min. PFS was given to slices at 2-min intervals throughout the recording except for 16 min immediately after SNP application. *b*, Effects of SNP alone on the K^+ response. PFS was ceased for 16 min during SNP application and for the following 16 min. *c*, Long-term depression evoked by SNP plus PFS in a 3AP slice. *d*, Changes in the K^+ response resistant to kynurenic acid (KYN, 10 mM) before and after long-term depression evoked by SNP plus PFS. Note the peak K^+ responses during the first and second KYN application were similar although they were considerably different immediately before the application.

First, excitatory post-synaptic currents in Purkinje cells following climbing-fibre stimulation are blocked by CNQX in slices obtained from young rats¹² or in cultured slices¹³, but the NO response in slices obtained from adult rats is not blocked by CNQX. Second, basket cells are stained with an antibody to an NO synthase¹⁴, but Purkinje cells are not. In spite of this uncertainty, the endogenous NO source must be located in the molecular layer because the onset of NO release following initiation of white-matter stimulation was as fast as that of probe currents induced by a step increase in NO concentration at the tip of NO probes.

Haemoglobin blocked long-term depression almost completely at 1 μ M, but about 50% of endogenous NO release in normal medium was recorded in the medium containing 10 μ M haemoglobin. This discrepancy suggests that the distance between the NO source and the effective target of NO to evoke

long-term depression may be longer than that between the NO source and the NO probe. Possible target sites of NO are the dendritic spines of Purkinje cells where G-kinase¹⁵ is found, or Bergmann glia which are located around the synapse between parallel fibres and dendritic spines and contain cGMP at high concentrations¹⁶. NO has been assigned a unique role as an intercellular messenger in the nervous system because it can diffuse a long distance and penetrate cell membranes freely¹⁷. Our study supports this expectation.

Quisqualate induces long-term desensitization of ionotropic glutamate receptors of non-NMDA type in Purkinje cells, and this desensitization is blocked by haemoglobin or NMMA (ref. 18). It is probable that the endogenous NO release shown in this study is required for common molecular mechanisms of long-term depression and the quisqualate-induced desensitization. □

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Rejection of class I MHC-deficient haemopoietic cells by irradiated MHC-matched mice

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IRRADIATED MHC-heterozygous mice often reject bone marrow cells transplanted from one of the homozygous parental strains, a phenomenon ('hybrid resistance') that appears to violate the laws of transplantation^{1,2}. Rejection of parental and allogeneic marrow cells also differs from conventional T cell-mediated rejection mechanisms as it is effected by NK1.1⁺ cells³⁻⁵. To account for the unusual specificity of bone marrow rejection, it has been proposed that NK1.1⁺ cells destroy marrow cells that fail to express the full complement of self MHC class I (MHC-I) molecules⁵. We show here that NK1.1⁺ cells in normal mice reject haemopoietic transplants from mice that are deficient for normal cell-surface MHC-I expression because of a targeted mutation in the β_2 -microglobulin gene⁶⁻⁹. These findings demonstrate that deficient expression of MHC-I molecules renders marrow cells susceptible to rejection.

To establish whether haemopoietic cells from MHC-I-deficient mice can proliferate in irradiated normal mice, we transferred bone marrow cells from homozygous β_2 -microglobulin (β_2m) mutant ($-/-$) mice of the 129/Sv (129) strain to groups of irradiated normal mice. The $-/-$ 129 marrow proliferated very poorly in either fully matched $+/-$ 129 hosts or in H-2-matched B6 hosts (B6 and 129 strains are both of the H-2^b haplotype, although they differ in their Qa/Tl region gene composition¹⁰; L. Flaherty, personal communication). By contrast, marrow cells from $+/-$ 129 mice proliferated at least 30-fold more in either host (Fig. 1a). We obtained similar results when (B6 $\times$ 129) F2 and F3 mice, also H-2^b, were used as $+/+$ or $-/-$ donors (Fig. 1b). Thus β_2m -mutant marrow cells fail to proliferate significantly in irradiated MHC-matched hosts. The F2 and F3 mice were used in subsequent experiments as there were more of them available than 129 $-/-$ mice (see Fig. 1 legend).

Mutant haemopoietic cells not only failed to proliferate in irradiated normal mice, they failed to protect these mice from the effects of lethal irradiation. Irradiated B6 mice inoculated with 5×10^6 mutant bone marrow cells (depleted of T cells) (Fig. 2a) or 7×10^6 mutant fetal liver cells (Fig. 2b) died 8 to 16 days later. The same doses of haemopoietic cells from wild type $+/+$ or heterozygous $+/-$ littermates resulted in long-term survival of irradiated B6 recipients (Fig. 2a and b, and data not shown). A titration experiment (not shown) revealed that all lethally irradiated B6 mice were protected by 1.5×10^6 $+/-$ fetal liver cells but that 80% died with even 3×10^7 $-/-$ fetal liver cells. Also, 5×10^6 $-/-$ H-2^b fetal liver failed to protect MHC-mismatched B10.BR mice from lethal irradiation, but these animals were protected by the same dose of $+/-$ fetal liver cells (Fig. 2e).

It is unlikely that T cell-depleted bone marrow cells or fetal liver cells from the mutant mice exert a lethal graft-versus-host reaction against the recipients, because irradiated recipients that received a 1/1 mixture of $-/-$ and $+/+$ marrow cells survived for at least 42 days (Fig. 2a). Analysis of cells repopulating these recipients revealed that lymph node, spleen and bone

marrow cells were of wild-type origin, as shown by cell-surface staining (Fig. 3a) and Southern blotting (Fig. 4a). These data indicate that mutant haemopoietic cells are unable to repopulate the irradiated wild-type recipients.

It has been shown that pretreatment of mice with a monoclonal antibody reactive against the NK1.1 marker present on some 3% of normal spleen cells eliminates NK1.1⁺ cells from mice¹¹ and prevents rejection of allogeneic bone marrow transplants^{3,5}. As shown in Fig. 2c, pretreatment of B6 mice with anti-NK1.1 monoclonal antibody before irradiation and inoculation with $-/-$ fetal liver cells prevented subsequent mortality; control recipients not pretreated with antibody died 7-12 days after transplantation. Most cells repopulating the lymph nodes ($>80\%$; Fig. 3b), spleen and bone marrow (Fig. 4b) of anti-NK1.1-pretreated animals were of mutant origin. These data indicate that there is no intrinsic defect in the capacity of $-/-$ stem cells to repopulate the haemopoietic system, but rather that normal MHC-matched mice reject the mutant cells. The simplest interpretation is that host NK1.1⁺ effector cells 'recognize' and destroy MHC-I-deficient donor cells. These effector cells are either absent or inactive in $-/-$ mice, because irradiated $-/-$ mice survive following transplantation of $-/-$ fetal liver cells (Fig. 2d). Irradiated $-/-$ mice also survive following transplantation of $+/+$ fetal liver cells (data not shown).

Although the rejection of wild-type allogeneic haemopoietic cells can be overcome by 5×10^6 donor cells, most recipients die with even 3×10^7 $-/-$ cells (not shown). This suggests that rejection of MHC-I-deficient cells is more rigorous than rejection of allogeneic cells. The effector cells mediating allogeneic and hybrid resistance show many similarities with natural killer cells, including their ontogeny, radiosensitivity and shared NK1.1⁺ phenotype^{3-5,12,13}. But the specificity of natural killer cells, which lyse certain tumour target cells with no evidence of MHC-allele specificity¹⁴, does not correspond generally to the specificity of bone marrow cell rejection. To add to the confusion, other studies suggest that bone marrow rejection is

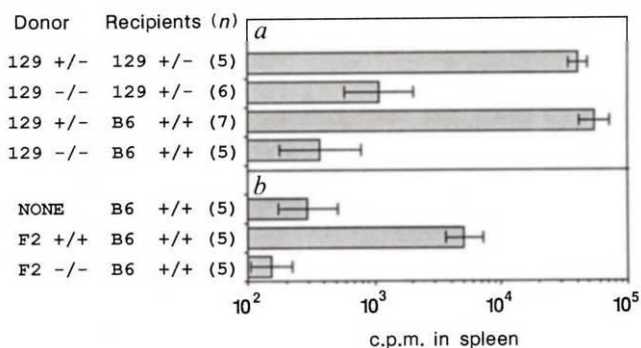


FIG. 1 Bone marrow cells from $-/-$ mice fail to proliferate in irradiated, MHC-matched mice. We use the incorporation of ^{125}I -labelled 5-iodo-2'-deoxyuridine (^{125}I -UdR) into donor marrow-derived cells in the spleens of irradiated recipients 5-6 days after transfer as a standard assay to determine the extent of donor cell proliferation^{1,3}. a, Strain 129 ($+/-$) and B6 ($+/+$) mice were irradiated (940 rads from a ^{137}Cs source at 100 rads per min) and then inoculated intravenously (i.v.) with 5×10^6 bone marrow cells from $+/-$ or $-/-$ 129 strain mice. Five days later the mice were inoculated intraperitoneally (i.p.)-labelled with $3 \mu\text{Ci}$ ^{125}I -UdR. Isotope incorporated in the recipient spleens was determined the next day after extensive rinsing of the spleens with PBS. Data are presented as geometric means with the standard error of the mean. The number of recipients in each group is indicated (n). b, As $+/-$ 129 marrow proliferated as well in B6 hosts as in syngeneic hosts (a), there are no 129-strain genes in the F2 mice that prevent marrow engraftment in B6 mice. We used (B6 $\times$ 129)F2 and F3 mutant mice for all subsequent experiments. Irradiated (980 rads) B6 mice were inoculated i.v. with 2×10^6 bone marrow cells from $+/+$ or $-/-$ (B6 $\times$ 129)F2 mice. The bone marrow cells were depleted of T cells by treatment with anti-Thy-1 plus complement²⁵. Also shown is a control group that received no marrow cells. ^{125}I -UdR incorporation was determined as described above.

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mediated by a fraction of NK1.1⁺ cells that express $\alpha\beta$ T-cell antigen receptors (about 25% of splenic NK1.1⁺ cells in B6 mice; our unpublished data)^{4,15}, whereas earlier results with T- and B cell-deficient SCID mutant mice argue that a T cell is unnecessary¹³. Whether the effector cell is a natural killer cell or an NK1.1⁺ T cell is also unclear from our system, in which MHC-I-deficient haemopoietic cells are rejected.

The mechanism of rejection of MHC-I-deficient marrow may

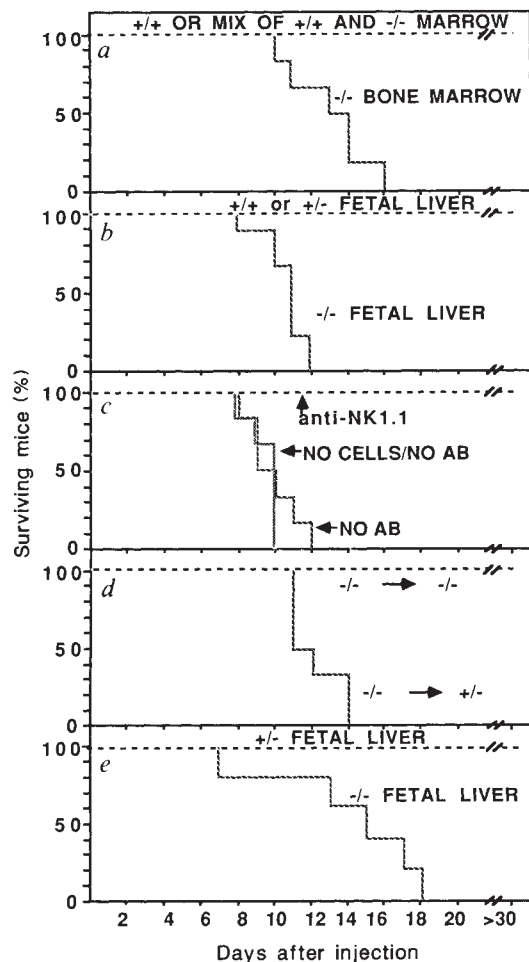


FIG. 2 Survival of irradiated mice inoculated with bone marrow or fetal liver. *a*, Irradiated groups of B6 mice ($n=6$) were inoculated with 5×10^6 +/- or -/- bone marrow cells depleted of T cells (see Fig. 1 legend), or a mixture of 5×10^6 of each. *b*, Irradiated B6 mice were inoculated with 7×10^6 fetal liver cells from -/- ($n=9$), +/- ($n=3$) or +/- ($n=6$) donors of embryonic age 17.5 days (E17.5). Fetal liver cells from individual donors were inoculated into three recipients. *c*, B6 mice were pretreated with NK1.1-specific monoclonal antibody ($n=7$), or not ($n=6$), before irradiation and inoculation with 4×10^6 -/- fetal (E17) liver cells. A control group received neither fetal liver cells nor antibody ($n=6$). In separate experiments, B6 mice ($n=6$) pretreated with irrelevant monoclonal antibody rejected -/- marrow normally (not shown). *d*, Irradiated +/- or -/- recipients ($n=6$ each) were inoculated with 5×10^6 -/- fetal (E16) liver cells. *e*, Irradiated B10.BR recipients were inoculated with 5×10^6 -/- ($n=5$) or +/- ($n=6$) fetal (E16) liver cells.

METHODS. Recipient mice were between 8 and 38 weeks old and of both sexes (no effect of sex was observed). They received 980 rads within hours of i.v. inoculation with cells. Mice were maintained on antibiotic water for 1–2 d before and 14 d after irradiation and reconstitution, except for the experiment in *d*, in which antibiotic water was first provided on the day of irradiation and reconstitution. Donors were (B6 $\times$ 129) F2, F3 and F2 $\times$ F3 animals which were genotyped by Southern blot analysis of tail DNA⁶. B6 recipients (*c*) were pre-depleted of NK1.1⁺ cells by i.p. injection of 200 μ g purified PK136 antibody¹¹ which is specific for NK1.1, 2 d before, and again 1 d before, irradiation and inoculation with fetal liver cells.

be related to that of haemopoietic cells that fail to express a subset of the host's MHC-I molecules, as in hybrid resistance. A 'missing self' model of marrow rejection has been proposed⁵, in which marrow cells are rejected that fail to be recognized by putative self MHC-I-specific receptors on NK1.1⁺ effector cells. The effector cells in this model would presumably bind to

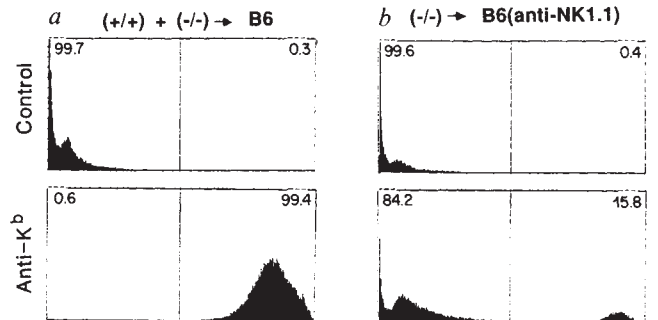


FIG. 3 *a*, Nearly all lymph node cells from irradiated B6 mice reconstituted 38 d earlier with a mixture of +/- and -/- marrow cells express K^b (see Fig. 2a legend for how these mice were produced). *b*, Most lymph node cells from B6 mice 35 d after pre-depletion of NK1.1⁺ cells, irradiation and reconstitution with -/- marrow, fail to express K^b (see Fig. 2c legend for how these mice were produced). The 15% of cells that express K^b presumably arise from 'radiation rebound' of host cells²⁶. **METHODS.** Lymph node cells were stained with M1/42 monoclonal antibody as described⁷. Control panels depict cells stained with only the second reagent (goat anti-rat IgG). M1/42 reacts with K^b of the H-2^b MHC-I molecules²⁷; positive staining requires $\beta 2m$ expression⁷. The shoulder on the negative population of stained cells from recipients of -/- marrow in *b* may be due to pick up of $\beta 2m$ from the $\beta 2m$ ⁺ recipients.

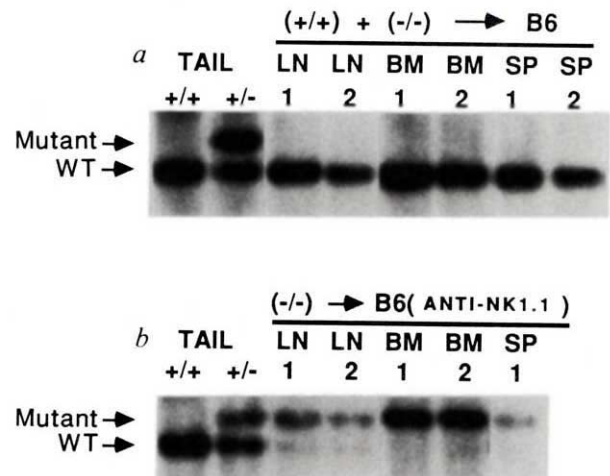


FIG. 4 Levels of donor cell reconstitution determined by Southern blot analysis of *EcoRI*-digested genomic DNA samples (5 μ g per sample) with a $\beta 2m$ -specific probe⁶. The upper band corresponds to the mutant $\beta 2m$ allele and indicates the presence of mutant cells, and the lower band corresponds to the wild-type (WT) allele. *a*, Genomic DNA of spleen (SP), bone marrow (BM) and lymph node (LN) cells from B6 recipients of a mixture of +/- and +/- bone marrow cells (see Fig. 2a legend). Two recipients (1 and 2) were tested 12 weeks after reconstitution. Note that not only the lymphoid (LN) compartment (see also Fig. 3a), but also the spleen and bone marrow compartments are virtually entirely of +/- origin. *b*, Genomic DNA samples of two B6 mice that were pre-depleted of NK1.1⁺ cells before irradiation and reconstitution with -/- fetal liver cells 7 weeks earlier (see Fig. 2c legend). Note that not only the lymph node cells (see also Fig. 3b), but also the bone marrow and spleen compartments are mostly of -/- origin. The faint wild-type band in the lymph node cell samples corresponds to a small fraction of B6 host cells that 'rebound' after irradiation (see Fig. 3b and Fig. 3 legend).

haemopoietic cells via receptors other than the MHC-I-specific receptors, yet reject only those cells that fail to engage their MHC-I-specific receptors. This model accounts for several observations, including hybrid resistance and the striking finding that B6 mice transgenic for the D^d MHC-I gene reject B6 marrow grafts⁵. A second model has MHC-I proteins on the cell surface masking non-MHC-I antigens that serve as targets for marrow graft rejection. This 'masking' model was proposed in a different context¹⁶⁻¹⁸, to account for the finding that loss of MHC-I expression by tumour cell lines augments their sensitivity to natural killer cells¹⁶⁻²⁰. The 'missing self' model can also explain these results. Finally, it has been proposed that haemopoietic cells express target antigens encoded by allelically variable recessive genes (*Hh-1* genes) in the MHC that are distinct from conventional MHC genes^{21,22}.

Our data on rejection of MHC-I-deficient marrow are most consistent with the 'missing-self' or 'masking' models. Either could have evolved to eliminate variant cells that lose MHC-I expression by mutation or as a result of viral infection²³, thereby evading conventional cytotoxic T lymphocyte immunity^{5,20,24}. Because it is hard to reconcile all marrow rejection data with any one model, it is possible that rejection of haemopoietic cells by NK1.1⁺ cells involves several mechanisms.

It is interesting that $-/-$ mice fail to reject their own marrow, as evidenced by their lack of haemopoietic deficiencies, and by the experiment shown in Fig. 2d. One interpretation of this is that the definition of 'self' with respect to marrow transplantation is determined by the environment in which the effector cells mature, by a tolerance-inducing and/or positive-selection process. Differentiation of the effector cells mediating rejection may require interaction with MHC-I molecules, as we found for CD8⁺ mature T cells, which fail to develop in $-/-$ mice⁷. We

are currently investigating the role of MHC-I expression in the development of natural killer cells and its influence on the rejection of allogeneic marrow transplants. □

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Expression of anti-DNA immunoglobulin transgenes in non-autoimmune mice

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SELF-REACTIVE B cells can be regulated by either deletion or inactivation¹. These manifestations of self-tolerance have been dramatically shown in transgenic mice in which the number of self-reactive cells has been artificially expanded^{2,3}. We have now extended these models to ask if B-cell tolerance as described for non-disease-associated antigens also operates for the targets of autoimmunity. The target we have chosen is DNA. Anti-DNA antibodies are diagnostic of certain autoimmune syndromes in humans and are a characteristic of the murine model of systemic autoimmunity, the MRL/lpr mouse⁴. Antibodies to both single-stranded and double-stranded DNA have been implicated in disease^{5,6}. By generating anti-DNA transgenic mice, we have addressed the question of whether DNA-specific B cells are regulated in normal (non-autoimmune) mice. We indeed found that most transgenic B cells bind DNA, yet we failed to detect secreted anti-DNA. We suggest that as a consequence of their self-reactivity these B cells are developmentally arrested.

Transgenic mice were constructed using the heavy (H) chain variable (VH) gene of a monoclonal anti-DNA antibody, 3H9, which arose spontaneously in a diseased MRL/lpr mouse⁷. The

VH gene coding for 3H9 is repeatedly used in anti-DNA antibodies from this autoimmune strain where it is associated with a variety of light (L) chains including representatives of the V κ 4, V κ 8, V κ 9, and V κ 23 groups (ref. 8 and J. E., manuscript in preparation). The variety of L chains which can combine with the VH3H9 to yield an anti-DNA antibody has been extended by transfection to include members of V κ 3 and V κ 21 groups and V λ 1 (ref. 30). Most of these VH3H9/VL combinations result in antibodies that bind both single-stranded (ss) and double-stranded (ds) DNA^{8,30}, but one combination, VH3H9/V κ 8, binds only ssDNA. The dominant role of the 3H9 H chain in determining DNA binding allows us to study the regulation of different kinds of anti-DNA antibodies. For example, by mating the VH3H9 transgenic to a pre-existing V κ 8 transgenic (C.C., S.A.C., J. J. Mackle, W. U. Gerhard and M.W., manuscript submitted) we would expect to generate a monospecific anti-ssDNA transgenic mouse³⁰. In a VH3H9-only transgenic, in which the VH3H9 is combined with many endogenous L chains, we would expect both anti-ssDNA antibodies as well as antibodies that bind both ss and dsDNA. This report focuses on two transgenic lineages: the VH3H9 H-chain-only transgenic and the progeny (VH3H9/V κ 8) of mating this VH3H9 transgenic to the V κ 8 transgenic.

The H-chain transgene dominates the preimmune repertoire in both the VH3H9 and VH3H9/V κ 8 transgenics, as shown in three separate analyses. First, VH3H9 messenger RNA comprises the majority of splenic immunoglobulin mRNA (Fig. 1b). Second, lymphocytes from these mice lack surface IgD (Fig. 2a). As the VH transgene construct does not include the IgD constant region gene (Fig. 1a), had IgD expression been observed it would have originated from endogenous genes. Finally, few, if any, complete endogenous immunoglobulin gene rearrangements are found among hybridomas from either the VH3H9 or VH3H9/V κ 8 transgenics (Table 1). Hybridomas derived from VH3H9/V κ 8 also showed little endogenous κ chain rearrangement (Table 1).

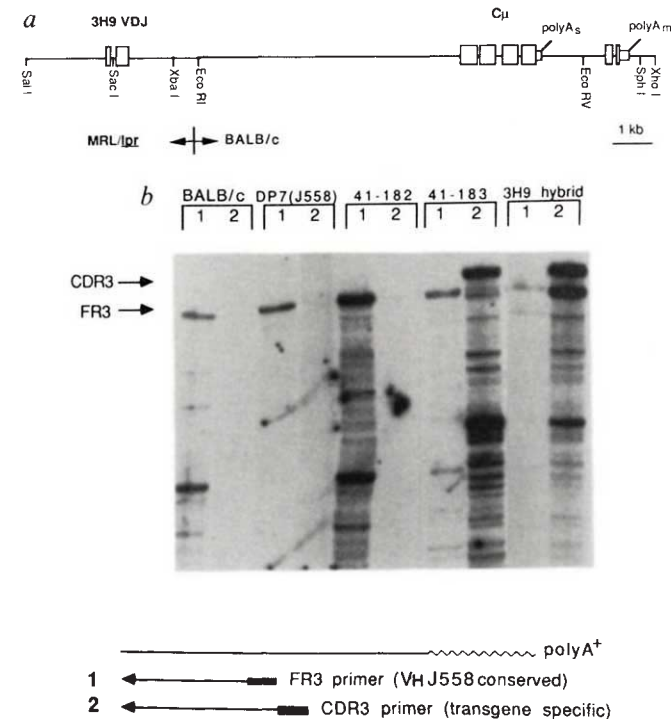


FIG. 1 The Vh3H9 transgene construct and its transcription. *a*, The restriction map of the Vh3H9 construct. The productive H-chain gene from the 3H9 MRL/lpr anti-DNA hybridoma was joined to the BALB/c IgM constant region²². This construct includes the secreted and membrane exons and 3' untranslated sequences of IgM but does not include IgD. Unique restriction enzyme sites and enzymes cutting only once in the VDJ region are indicated. *b*, Expression of the transgene was evaluated by primer extension analysis of spleen cell poly(A)⁺ RNA. Identical amounts of poly(A)⁺ RNA were annealed to the framework region (FR3) primer, homologous to a conserved region of Vh J558 genes (lanes 1), or the complementarity-determining region (CDR3) primer, homologous to the junctional diversity region of the Vh3H9 transgene (lanes 2). In a normal mouse the J558 family comprises the majority of splenic immunoglobulin mRNA (~50%)²³, yet the Vh3H9-specific RNA is undetectable: no signal in CDR3 lanes is seen with BALB/c RNA, RNA from a non-transgenic littermate (41-182) or from a hybridoma that uses a different J558 gene (DP7). Extension with both primers is seen only when RNA from a transgene-positive mouse (41-183) or RNA from the 3H9 hybridoma is used. The majority (62%) of J558 mRNA in transgenic spleen 41-183 is transcribed from the transgene. Therefore, at least 31% of total mRNA is 3H9 derived. This is certainly an underestimate. We think it is unlikely that the Vh3H9 transgene exerts its influence just on the J558 family. Furthermore, plasma cells contribute disproportionately to splenic immunoglobulin mRNA levels²⁴ and may overrepresent endogenous Vh genes.

METHODS. *a*, The productively rearranged 3H9 VDJ region was isolated from a size-enriched λ phage library as a 4.4-kilobase *EcoRI* fragment. Subsequently, the 5' *EcoRI* site was removed using exonuclease III and mung bean nuclease, and a *SalI* restriction site linker was inserted. The 3H9 VDJ region was joined to the 11.6-kb *EcoRI*-*XhoI* C μ fragment from BALB/c²². The *SalI*-*XhoI* fragment containing the entire Vh3H9 construct was purified from plasmid sequences and microinjected into pronuclei of fertilized (C57BL/6 X SJL) embryos²⁵. Thirteen founder animals were obtained; the progeny of one of these (no. 41) is described in this report. *b*, Poly(A)⁺ RNA was prepared from spleens or hybridoma cells. RNA (3 μ g) was annealed to 5 pg ³²P-labelled oligonucleotides using either the FR3 (5'-TCTTGACAGT-AATAGATGCAGAGT-3') or the CDR3 primer and converted to cDNA. Reaction products were analysed by PAGE and autoradiography. Full-length cDNA was quantitated for each RNA sample by digitized image scanning of the FR3 and CDR3 lanes. The proportion of Vh3H9-derived message among the J558 transcripts in transgenic mice was calculated from the FR3/CDR3 cDNA signal ratio of 3H9 hybridoma transcripts (A), and the corresponding ratio of signals obtained using 41-183 splenic transcripts (B) according to the formula: $X = 1 - (B - A)/B$.

TABLE 1 Summary of transgenic hybridomas

	Vh3H9/Vk8	Vh3H9
(a) Immunoglobulin ⁺ total (%)	80	88
Transgene ⁺ /immunoglobulin ⁺ (%)		
Vh3H9	83	95
Vk8	93	NA*
H ⁰ /H ⁰ , H ⁰ /H ¹ , H ¹ /H ¹ , H ¹ /-†	16, 17, 12, 9‡	17, 33, 17, 18‡
K ⁰ /K ⁰ , K ⁰ /-§ (%)	83	NA*
(b) Specificity		
ssDNA (%)	84	52
dsDNA (%)	0	0
Cardiolipin (%)	84	30

Hybridomas were derived from LPS-stimulated spleen cells from both the Vh3H9/Vk8 and the Vh3H9 transgenics. One-hundred-and-sixteen hybridomas were analysed from the Vh3H9 transgenic and seventy-five from the Vh3H9/Vk8 transgenic. Immunoglobulin-secreting hybridomas were assayed for the presence of the transgene(s) and for the extent of endogenous immunoglobulin gene rearrangement. *a*, Vh3H9 transgene positive animals were first identified by Southern blot analysis and subsequently using polymerase chain reaction (PCR)¹⁶ with the following primers: 5'-CTGTCCAGGAAGTGCAGTAAGG-3' (Vh3H9 leader intron), and 5'-CATAAC-ATAGGAATATTTACTCCTCGC-3' (Vh3H9 CDR3). Vk8 transgene animals were identified using PCR with Vk8-specific primers: 5'-GGTACCTGTGGG-ACATTGTG-3' (Vk8 leader), 5'-AGCACCGAAGCTGAGAGG-3' (Vk8-JK5). Hybridomas were generated from Vh3H9/Vk8 and Vh3H9 transgenic spleen cells 3 days after an injection of 50 μ g LPS as described¹⁷. The presence of the transgene(s) and the extent of endogenous immunoglobulin gene rearrangement was determined using Southern blot analysis as described¹⁸. Messenger RNA sequence analysis was performed on three of the Vh3H9/Vk8 hybridomas and four of the Vh3H9 hybridomas, establishing that the transgenes are expressed and that the expressed transgene(s) are unaltered⁶. Messenger RNA primer extension analysis from 32 additional Vh3H9 hybridomas confirmed the use of the Vh3H9 transgene in 31 of the 32 hybridomas (data not shown). *b*, The hybridoma monoclonal antibodies were assayed for their ability to bind ssDNA, dsDNA, and cardiolipin. Only hybridomas which had retained the transgene(s) DNA are included. The secretion and specificity of the monoclonal antibodies was determined in the enzyme-linked immunoabsorbent assay (ELISA) essentially as described for dsDNA¹⁹, ssDNA²⁰, and cardiolipin²¹.

* NA, not applicable.

† Number of hybridomas with the following genotype: H⁰/H⁰, both endogenous heavy chain alleles are in germ-line configuration; H⁰/H¹, one allele is in germ-line configuration and the other is rearranged; H¹/H¹, both alleles are rearranged; H¹/-, one allele is absent, presumably owing to chromosome loss in the hybridoma, and the other is rearranged.

‡ 33% of the endogenous heavy chain rearrangements identified in the Vh3H9 hybridomas and 43% in the Vh3H9/Vk8 hybridomas have restriction fragments similar in size to those identified as incomplete, D to Jh rearrangements¹⁵.

§ K⁰/K⁰, both endogenous kappa alleles are in germ-line configuration; K⁰/-, one endogenous κ allele is in germ-line configuration, the other is absent, presumably as a result of chromosomal loss.

The overwhelming degree of endogenous gene exclusion predicts that the Vh3H9/Vk8 transgenic mice should essentially produce monospecific anti-DNA immunoglobulins. This prediction was tested by examining the specificity of spleen cell surface immunoglobulins as well as antibodies produced by hybrids derived from the transgenics. In these mice the majority (60-69%) of the IgM⁺ cells bound ssDNA (Fig. 2b). Similarly, most hybridomas derived from lipopolysaccharide (LPS)-activated spleen cells express anti-DNA antibodies (Table 1). Moreover, these antibodies have the features of the antibodies expressed by the Vh3H9/Vk8 transfectoma³⁰, in that they bind ssDNA and cardiolipin but not dsDNA. The frequencies of anti-DNA expression are substantially higher than those found in non-transgenic littermates (Fig. 2b) or among LPS hybrids from normal mice^{9,10}.

Despite the high frequency of anti-DNA B cells in the Vh3H9/Vk8 transgenics, anti-DNA serum titres are not higher than those found in normal mice (Fig. 3a). In this regard our mice are different from other immunoglobulin transgenics, such

as the anti-lysozyme and anti-H-2^k transgenics, which have high serum levels of the transgene-encoded antibody^{2,3}. That the VH3H9/V κ 8 mice show no increase in anti-DNA titres over the background levels observed in normal mice, and also display reduced serum IgM levels (Fig. 3b), suggests that the anti-DNA B cells are blocked in their ability to differentiate into antibody-secreting cells. In this respect the VH3H9/V κ 8 B cells are similar to the anti-lysozyme B cells in mice that also carry a transgene coding for the neo-self-antigen, hen-egg lysozyme¹¹. By analogy with the lysozyme/anti-lysozyme tolerance model, we propose that the anti-DNA B cells have encountered antigen, for example, DNA or DNA-protein complexes, and this encounter has left them functionally silent. It is possible that the inability to detect anti-DNA activity, along with the abnormally low level of IgM, is due to immune complexes and/or tissue deposits being formed between the transgenic antibody and self-antigen, thereby masking serum anti-DNA activity. We think this is unlikely because anti-DNAs are readily detectable in autoimmunity⁵. Future studies on the expression of the VH3H9/V κ 8 antibodies on the genetic background of the autoimmune mouse should resolve this issue.

Comparison with the lysozyme/anti-lysozyme transgenics shows a difference in phenotype of self-reactive B cells. Whereas B-cell anergy in the lysozyme/anti-lysozyme transgenics correlates with a low density of membrane IgM¹¹, this is not the case for the VH3H9/V κ 8 transgenic B cells. Here the B cells which bind ssDNA have a high density of surface IgM (Fig. 2b). This indicates that B-cell anergy is not always correlated with down-regulation of membrane IgM.

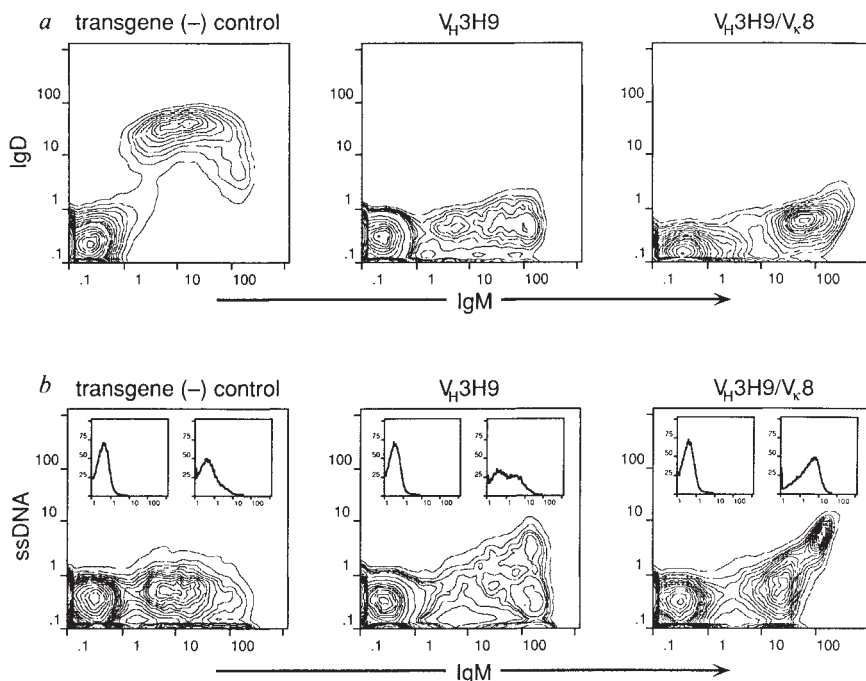
The VH3H9-only transgenic mice provide a model for survey-

ing the potential range of specificities that result from pairing VH3H9 with the endogenous L-chain pool. As discussed, natural and transfectoma antibodies in which this H chain is paired with a variety of L chains bind both ds and ssDNA, and when paired with other L chains, bind only ssDNA³⁰. Specificity analysis of VH3H9-only transgenic spleen cells shows that 63% (range 38–86%) of the IgM⁺ B cells bound ssDNA, and that the remainder did not bind DNA at all (Fig. 2b). This latter category shows that association with certain L chains can abolish DNA binding. Similarly, hybridomas from the VH3H9 mice express both DNA-binding and non-DNA-binding antibodies (Table 1). Although there are a variety of L chains which in combination with the VH3H9 can bind both ss and dsDNA³⁰, we found no dsDNA-binding monoclonal antibodies. One possible explanation is that B cells with this specificity (as opposed to anti-ssDNA only) are deleted. Alternatively, anti-dsDNA lymphocytes may exist but escape our notice because they are expressed in a population of cells refractory to LPS activation¹² and/or fusion. If this population is deleted or nonfunctional then we would expect the remaining V κ repertoire to be reduced. In fact it is: mRNA sequence analysis of L-chain genes from 32 hybrids reveals use of a limited variety of L chains in both the non-DNA-binding and the ssDNA-binding hybrids (M.Z.R., J.E. and M.W., manuscript in preparation).

Fifty-two per cent of the VH3H9 hybrids secrete anti-ssDNA antibodies, a frequency similar to the average number of DNA-binding spleen cells (Fig. 2b), yet, like the VH3H9/V κ 8 transgenics, the VH3H9 mice show no increase in anti-DNA serum titres above those seen in normal mice (Fig. 3a). Therefore, anergy applies not just to the VH3H9/V κ 8 anti-DNAs, but also

FIG. 2 Fluorescence-activated cell sorter (FACS) analysis of spleen cells. *a*, Cell-surface analysis shows that splenic cells from a non-transgenic animal express both IgM and IgD isotypes²⁶ whereas cells from the VH3H9/V κ 8 and VH3H9 transgenic mice express IgM alone. On the basis of the distribution of B220, Ly-1, Thy-1, CD3, CD4 and CD8, the B- and T-cell composition of these transgenics is equivalent to normal mice (data not shown). Non-transgenic animals express a range of IgM and IgD cell-surface densities. The majority of the IgM⁺ cells in the VH3H9/V κ 8 mice have a high density of surface IgM. Although the VH3H9 mice also have many cells with a high IgM surface density, there are also cells with lower levels of IgM. *b*, Spleen lymphocytes were analysed for cell-surface binding to ssDNA and IgM. The inset panels display DNA binding on the x-axis and relative cell number on the y-axis. Threshold for detection in the FACS DNA assay is dependent on surface immunoglobulin density. Therefore, only IgM⁺ cells (defined as >50 fluorescence units on the IgM scale) were quantitated. The left inset shows DNA binding in the IgM⁺ cell population and the right inset is for the IgM⁺ cell population. Non-transgenic animals showed very little binding (8–17%) to ssDNA in either cell population. In the example shown here, 67% of IgM⁺ cells in the VH3H9/V κ 8 transgenic bound ssDNA (range 60–69%). The VH3H9 transgenics have two populations of IgM⁺ cells: 40% that bind ssDNA and 60% that do not. There was considerable variation in the percentage of DNA⁺ cells among VH3H9 transgene animals, range 38–86%. All the DNA-binding cells in both transgenics were IgM⁺, ruling out the possibility that the (non-immunoglobulin) DNA receptor that has been reported on splenic T cells was being detected²⁷.

METHODS. *a*, Offspring from matings between F₁ (VH3H9⁺C57BL6/J $\times$ MRL⁺/+) and V κ 8⁺ BALB/c mice were genotyped as described in Table 1. Spleen cells from animals either negative for both transgenes, positive for both (VH3H9/V κ 8), or positive for only the VH3H9 transgene were stained simultaneously with fluorescein-conjugated anti-IgD^a allotype (AMS 15.1), biotin-anti IgD^b allotype (AF6-122) (revealed by Texas-Red-avidin) and allophycocyanin (APC)-anti-IgM (331.1.2), and then analysed using a dual-laser FACSTAR⁺ (refs 28, 29). A total of eight negative, nine VH3H9, and



five VH3H9/V κ 8 animals were analysed. The examples shown are stained with anti-IgD^a; the anti-IgD^b showed no staining for this set of mice. In all FACS analyses, only cells falling within normal lymphocyte values for both forward and large-angle scatter were included (eliminating granular and dead cells). Propidium iodide was also included to exclude dead cells. Staining intensities are expressed in arbitrary fluorescence units on four-decade logarithmic scales. *b*, Spleen cells were stained simultaneously with APC-anti-IgM and 10 μ g biotin-ssDNA (revealed by Texas Red-avidin) and analysed by FACS. Biotinylated DNA was prepared as previously described¹⁹. Independent experiments using the following sources of DNA gave similar results: S1 or mung-bean nuclease treated sonicated salmon sperm DNA; S1 or mung-bean nuclease-treated *Xba*I-digested λ phage DNA; S1-treated *Hae*III-digested salmon sperm DNA. DNA was prepared by heating to 95°C for 5 min and then rapidly chilled before use.

to the variety of anti-DNA antibodies formed by V_H3H9 plus endogenous L chains. In contrast to the V_H3H9/V_κ8 mice, the V_H3H9 mice have near normal levels of serum IgM (fig. 3b). We presume that the population of non-DNA-binding cells, identified in Fig. 2b and Table 1, is responsible for the normal levels of serum IgM.

These results show that anti-DNA antibodies are regulated in normal (non-autoimmune) animals. Most B cells in V_H3H9/V_κ8 and roughly half in the V_H3H9 transgenics are specific for ssDNA, but they apparently do not differentiate into immunoglobulin-secreting cells. As these B cells retain a high density of surface IgM, the immunoglobulin on these cells may be uncoupled from the immunoglobulin signalling pathway^{13,14} rendering them functionally silent. Hybridomas from the V_H3H9 transgenics have suggested an additional mechanism of regulat-

ing anti-DNA B cells. Here we find anti-ssDNA antibodies but fail to find anti-dsDNA antibodies.

Most importantly, the results demonstrate that B-cell tolerance to DNA is manifested in ways similar to those described in experimental models of tolerance¹⁻³. This observation, that normal mice regulate disease-associated antibodies, implies that autoimmune disease results from a breakdown of regulation of autoantibody expression. □

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Expression of recombinant dystrophin and its localization to the cell membrane

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DUCHENNE'S muscular dystrophy (DMD) is an X-linked progressive myopathy caused by a defect in the DMD gene locus^{1,2}. The gene corresponding to the DMD locus produces a 14-kilobase (kb) messenger RNA that codes for a large cytoskeletal membrane protein, dystrophin^{3,4}. DMD and Becker's muscular dystrophy are the consequences of dystrophin mutations^{4,5}. The exact biological function of dystrophin remains unknown but it has been demonstrated that it is localized to the cytoplasmic face of the cell membrane and has direct interaction with several other membrane proteins^{6,7}. We report here the synthesis of a 14-kb full-length complementary DNA for the mouse muscle dystrophin mRNA and the expression of this cDNA in COS cells. The recombinant

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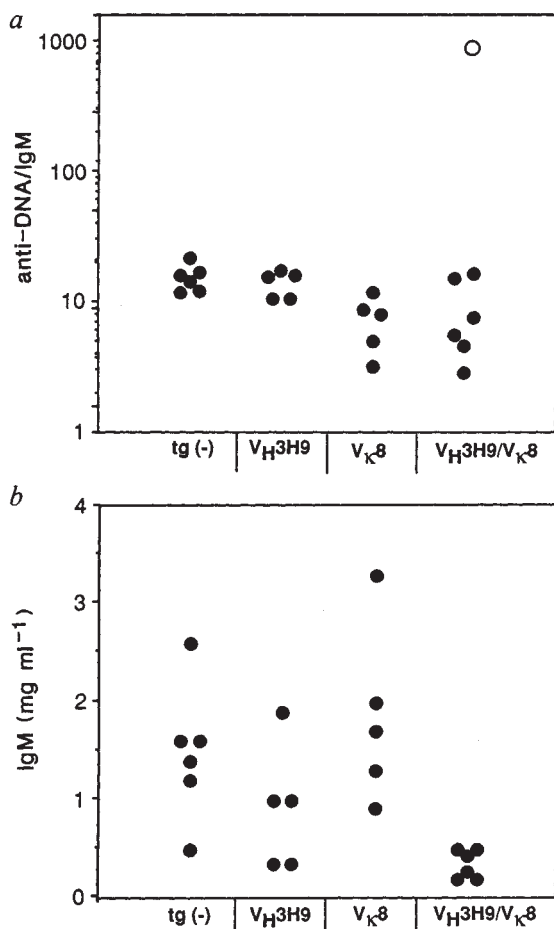


FIG. 3 Relative anti-DNA titres and IgM concentrations in serum from transgene-negative (tg^{-/-}) littermates and V_H3H9, V_κ8 and V_H3H9/V_κ8 transgenics showing: *a*, at a given IgM concentration there is no increase in anti-DNA titres in the transgenics above the background observed in non-transgenic mice. ○, Value obtained from one of the LPS-induced V_H3H9/V_κ8 hybrids at the equivalent IgM concentration. Each symbol represents values obtained for a single mouse. *b*, IgM levels in the non-transgenic, V_H3H9 and V_κ8 mice are similar whereas those in the V_H3H9/V_κ8 mice are depressed. METHODS. Offspring from matings between F₁ (V_H3H9⁺ C57BL6/SJL × MRL^{+/+}) and V_κ8⁺ BALB/c mice were assayed as described in Table 1 for the presence of transgenes and bled between 8 and 16 weeks of age. Sera were diluted in 1% BSA in PBS and assayed by ELISA for binding to polyspecific anti-immunoglobulin goat antibodies or heat-denatured calf thymus DNA (50 µg ml⁻¹) absorbed on protamine sulphate (200 µg ml⁻¹) coated plates²⁰. Anti-DNA titres represent dilutions required to achieve absorbance of 0.1. Standard IgM concentrations were prepared by dilution of purified antibodies from the murine myeloma 3741. Antibody levels were measured using an ELISA plate reader at 405 nm following incubations with goat anti-mouse IgM-conjugated alkaline phosphatase and substrate.

dystrophin is indistinguishable from mouse muscle dystrophin by western blot analysis with anti-dystrophin antibodies and was shown by an immunofluorescent technique to be localized in the cell membrane. Our successful construction of a functional full-length cDNA opens opportunities for the study of structure and function of dystrophin and provides an opportunity to initiate gene therapy studies.

We report a simple strategy by which the full-length mouse dystrophin cDNA was cloned and expressed in COS cells. The 14-kb dystrophin cDNA was cloned in two large fragments using a λ bacteriophage cloning vector with a 9-kb insert limit. Two cDNA libraries (A and B) were constructed with dystrophin-specific primers localized to the 3' end and the other at nucleotide position starting at 7,858. The two libraries were screened sequentially with three mouse dystrophin cDNA clones localized at the 5', middle and 3' end of the mRNA to identify large overlapping clones. From the A library a clone designated DMD 5', which has a 7.8-kb insert, and from the B library a clone designated DMD 3', with an 8.0-kb insert, were isolated. Clones DMD 5' and DMD 3' overlapped and shared a unique *Kpn*I site which was used to generate the full-length dystrophin cDNA. This was then cloned into the *Not*I site of the bluescript vector pSK⁻ giving rise to the clone pCCL-DMD (Fig. 1). Sequencing

of the 5' and 3' ends and restriction mapping indicate that the clone is of full length. Further characterization of this clone by the polymerase chain reaction indicates that it is the major muscle isoform⁸.

To determine whether this full-length cDNA construct is authentic, the dystrophin cDNA was excised from pCCL-DMD by *Not*I restriction digestion and cloned into the *Sma*I site of the mammalian SV40 expression vector pSVL to generate pSVL-DMD. Transfection of pSVL-DMD into COS cells was carried out by electroporation. Transient expression of the pSVL-DMD was determined 72 h later by western blot analysis with anti-dystrophin antibodies. As shown in Fig. 2, western analysis revealed the presence of a polypeptide of relative molecular mass 430,000 (430K), found in normal C57 mouse muscle, in the COS cell extracts from pSVL-DMD-transfected cells, but not in *mdx* mouse muscle, control COS cells or COS cells electroporated with the pSVL vector. Therefore the recombinant dystrophin is the same size as the muscle dystrophin.

To establish the authenticity of the dystrophin cDNA, the localization of the expressed recombinant dystrophin was determined by immunofluorescence. Expression of dystrophin from pSVL-DMD was not high enough in the cells for immunofluorescent localization. To achieve very high levels of recombinant dystrophin expression, the dystrophin cDNA was cloned into the pcDL-SR α 296 vector⁹ to generate pSR α -DMD. The SR α promoter is 10–30-fold more active than the SV40 promoter-enhancer unit⁹. Immunofluorescent analysis with anti-dystrophin antibodies and secondary FITC-antibodies was carried out on the COS cells 72 h after electroporation with pSR α -DMD. In the absence of DNA, none of the cells showed any fluorescence (Fig. 3a). About 3–5 per cent of the cells

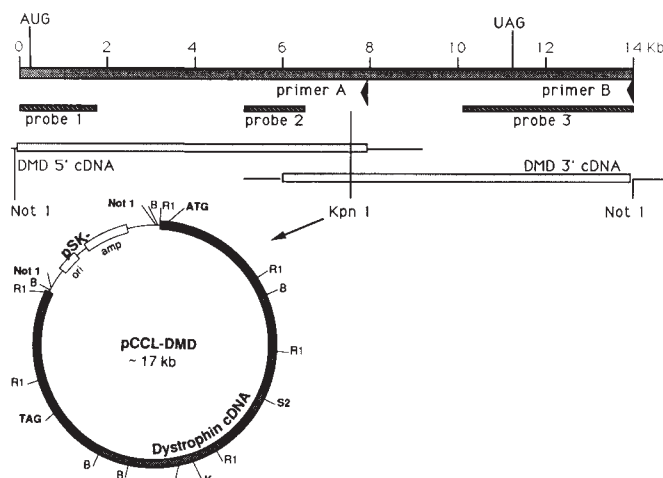


FIG. 1 Cloning and construction of pCCL-DMD. The top bar represents the full-length dystrophin mRNA. The location of the dystrophin primers A (5'-GTCTAGCTCTTGATTGCTGG-3') and B (5'-TTGTAACATAACTGCGTGGT-3') and the three probes designated 1, 2 and 3 spanning nucleotides 7–1834, 5,079–6,546 and 10,381–polyA respectively, are represented by the arrows and middle bars, respectively. One cDNA library was constructed by priming mRNA with a 22-mer oligonucleotide (A) whose sequence corresponds to the nucleotide position starting at 7,858. The second cDNA library was primed with a 20-mer oligonucleotide (B) whose sequence is adjacent to the mouse dystrophin poly(A) sequence. The A library was screened sequentially with probes 1 and 2 and library B was screened sequentially with probes 2 and 3. Both clones were then autoexcised from λ ZAP to give rise to their bluescript plasmid forms¹⁸. The bottom bars show the location, size and the unique *Kpn*I restriction site of the clones DMD 5' and DMD 3' which were spliced together and subcloned into pSK⁻ to generate pCCL-DMD. Restriction sites within pCCL-DMD are *Bam*HI(B), *Eco*RI(R1), *Sac*II (S2) and *Kpn*I(K).

METHODS. The construction of the dystrophin-specific primed cDNA libraries was carried out with 4 μ g poly (A)⁺ mouse skeletal muscle RNA using 2 pmol of either primer A or B for the first strand cDNA synthesis with 200 U Molony murine reverse transcriptase (BRL) and 50 nmol dNTP at 37 °C for 1 h. The second-strand cDNA synthesis and subsequent steps for the library construction were carried out as described.¹⁹ The λ ZAP cloning vector was obtained from Stratagene. The entire length of the mouse dystrophin mRNA is 13,815 bases excluding the poly(A) sequence and the 5' end of pCCL-DMD corresponds to nucleotide position 30 of the mouse cDNA sequence²⁰. The ATG start site is at nucleotide position 221–223 and the TAG stop codon is at position 11,255–11,257.

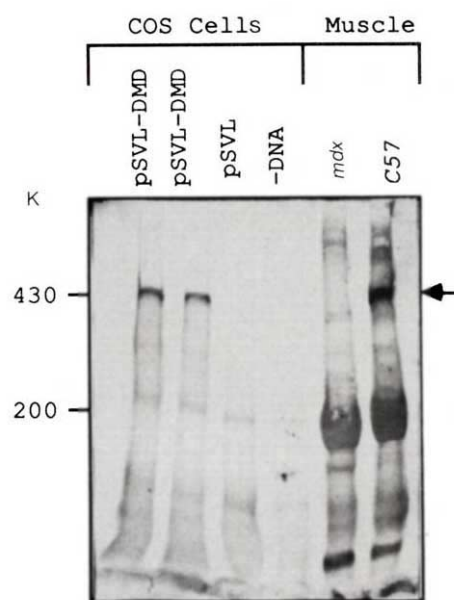


FIG. 2 Western blot analysis for dystrophin expression. The first four lanes are COS cell extracts prepared from cells that have been electroporated with the pSVL-DMD (20 μ l and 10 μ l), pSVL (20 μ l) and no DNA (20 μ l). The two lanes on the right are muscle extracts from C57 normal and *mdx* mice. The arrow indicates the location of dystrophin protein in the C57 mouse muscle extract which is absent in the *mdx* mouse muscle extract.

METHODS. Electroporation of 10^7 COS (African green monkey kidney) cells was carried out at 960 μ F and 200 V in 0.8 ml of PBS buffer using 15 μ g DNA. Muscle was obtained from the hind legs of either C57 normal or *mdx* dystrophin-deficient mice. The tissue and cell extracts were prepared in SDS denaturation buffer before being separated on a 3.5% SDS-polyacrylamide gel and electroblotted onto a nitrocellulose membrane. The presence of dystrophin was determined using the alkaline phosphatase procedure with anti-dystrophin primary antibodies⁴ (preadsorbed with COS cell extract) and a biotinylated anti-sheep secondary antibody.

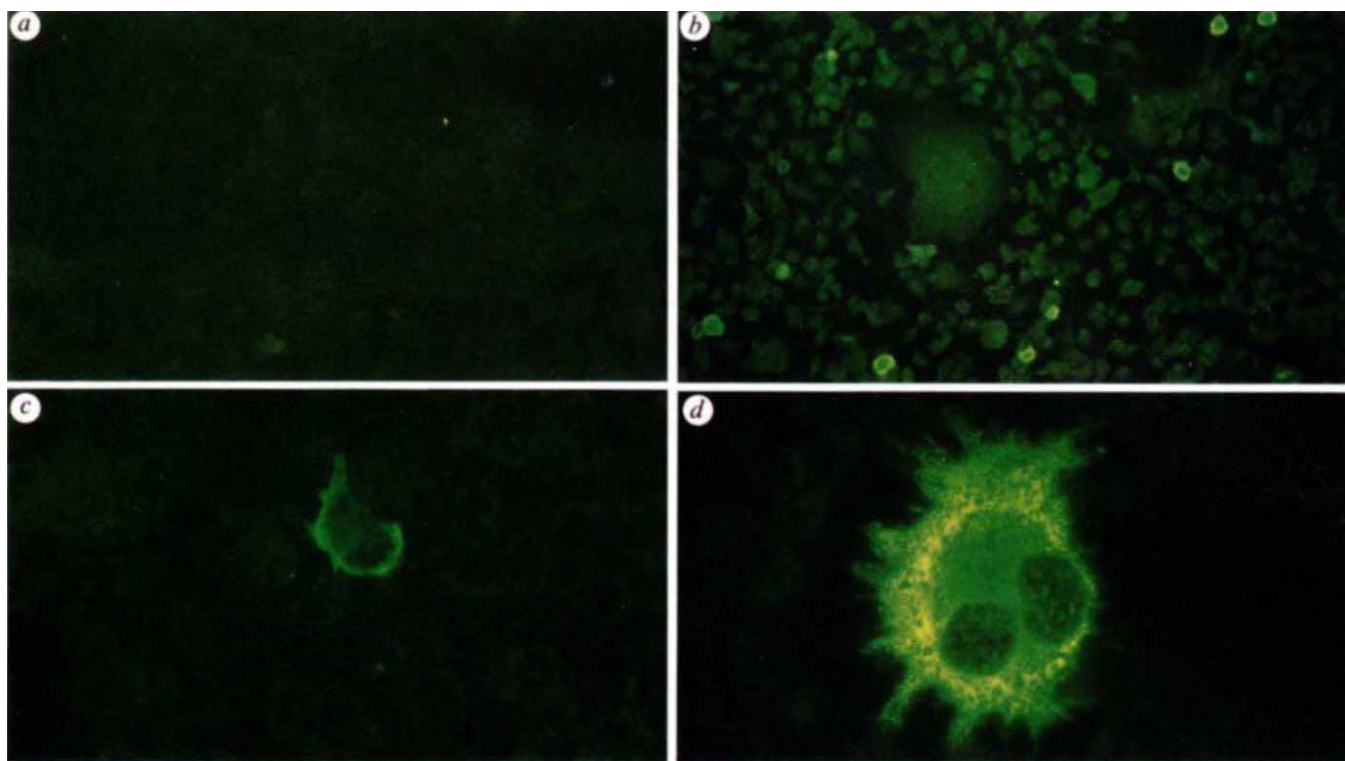


FIG. 3 Localization of recombinant dystrophin by immunofluorescent activity. Electroporation was carried out in the absence (a) or the presence (b-d) of 15 μ g pSR α -DMD on 10^7 COS cells. The cells were photographed in a Zeiss Axiophot microscope. Note that the region of immunofluorescence is predominantly around the rim of the cell.

METHODS. The electroporated cells were serially diluted into chamber slides and after 72 h washed twice with PBS and then fixed for 1 min in acetone.

The slides were washed in PBS containing 1% BSA for 15 min before the addition of the primary antibodies. The primary antibodies had been preadsorbed with acetone-fixed COS cells. After 2 h the slides were washed several times with PBS and incubated with a fluorescein isothiocyanate (FITC) anti-sheep IgG conjugate for 1 h. The slides were then washed overnight in PBS before being mounted in 10 mg ml⁻¹ *p*-phenylenediamine in 10% PBS and 90% glycerol.

electroporated with pSR α -DMD fluoresced intensely around their rim and slightly within their nucleus or cytosolic region (Fig. 3b-d). Immunofluorescence at the cell rim indicates that the expressed recombinant dystrophin is localized at the cell membrane. This, together with the expressed dystrophin being the same size as muscle dystrophin, implies that the full-length dystrophin cDNA is authentic.

These studies demonstrate a simple way of cloning and expressing a full-length dystrophin cDNA. Using specific dystrophin sequence primers we were able to synthesize large cDNA fragments from mouse muscle poly(A)⁺ RNA, reducing the number of necessary overlapping fragments to two. The approach is simpler than that described for the cDNA cloning and expression of the muscle ryanodine calcium receptor, a cDNA of similar dimension which requires 10 overlapping cDNA fragments to generate a full-length clone¹⁰. The technique by which this dystrophin cDNA was generated is applicable to cloning cDNAs of large structural proteins in muscle such as

nebulin (25 kb) and titins¹¹. Consistent with previous reports that dystrophin is a plasma membrane protein³⁻⁷, we also demonstrated that the expressed recombinant dystrophin is targeted to the cell membrane. Despite the normal level of dystrophin in kidney being only 1.6 per cent of that in muscle¹², the overexpressed dystrophin was still found predominantly in COS cell membranes. This suggests that the signal for membrane localization lies within the dystrophin protein domain. The DMD minigene should be useful not only to identify those protein domains responsible for membrane localization but also to help elucidate the role of dystrophin in intracellular calcium regulation¹³⁻¹⁵. The construct could also prove to be of use in correcting the dystrophin deficiency by gene replacement. Indeed, wild-type myoblast transfer into *mdx* mice results in expression of dystrophin¹⁶, and direct injection of DNA into myotubes of mice can result in transient expression of a reporter gene¹⁷. Collectively these studies offer the hope that gene transfer will be of therapeutic benefit to affected patients. □

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Metabolic stabilization of endplate acetylcholine receptors regulated by Ca^{2+} influx associated with muscle activity

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DURING formation of the neuromuscular junction, acetylcholine receptors in the endplate membrane become metabolically stabilized under neural control, their half-life increasing from about 1 day to about 10 days (see ref. 1 for review). The metabolic stability of the receptors is regulated by the electrical activity induced in the muscle by innervation²⁻⁴. We report here that metabolic stabilization of endplate receptors but not of extrajunctional receptors can be induced in the absence of muscle activity if muscles are treated with the calcium ionophore A23187. Acetylcholine receptor stabilization was also induced by culturing non-stimulated muscle in elevated K^+ with the Ca^{2+} channel activator (+)-SDZ202-791. Conversely, activity-dependent receptor stabilization is prevented in muscle stimulated in the presence of the Ca^{2+} channel blockers (+)-PN200-110 or D-600. Treatment of muscles with ryanodine, which induces Ca^{2+} release from the sarcoplasmic reticulum in the absence of activity, does not cause stabilization of junctional receptors. Evidently, muscle activity induces meta-

bolic acetylcholine receptor stabilization by way of an influx of Ca^{2+} ions through dihydropyridine-sensitive Ca^{2+} channels in the endplate membrane, whereas Ca^{2+} released from the sarcoplasmic reticulum is ineffective in this developmental process.

Experiments were performed on chronically denervated soleus muscles of male Sprague-Dawley rats. The metabolic stability of junctional and extrajunctional acetylcholine receptors (AChRs) was estimated from the rate of loss of specific radioactivity, as determined by quantitative autoradiography, from synaptic and extrasynaptic membrane after labelling the AChRs with [^{125}I] α -bungarotoxin⁴. At synapses in a steady state, AChR degradation is a first-order process¹. AChR stabilities were expressed in terms of apparent half-lives ($t_{1/2,\text{app}}$) of the AChRs, as in some experiments, some deviation of AChR degradation from a single exponential time course was observed (see legend to Fig. 1). The results of the experiments are summarized in Table 1.

After soleus muscles are denervated, the average metabolic half-life, $t_{1/2}$, of AChRs in the endplate membrane declines within about 2 weeks from 10–12 days to about 2 days; at the same time AChRs with a $t_{1/2}$ of about 1 day appear in the extrajunctional membrane (see ref. 1 for review). Metabolic stabilization of endplate AChRs could be induced when the chronically denervated muscles were stimulated in organ culture. Figure 1a, b shows endplates from pairs of contralateral soleus muscles of the same rat after 24 h of stimulation and subsequent labelling with [^{125}I] α -bungarotoxin. The muscle in Fig. 1a was fixed immediately after rinsing, that in Fig. 1b after 72 h of culture. Endplates from non-stimulated muscles are shown for comparison (Fig. 1g, h). Autoradiographic grain density, although initially similar, is lower in inactive than in active muscle after culture, suggesting that the stability of endplate

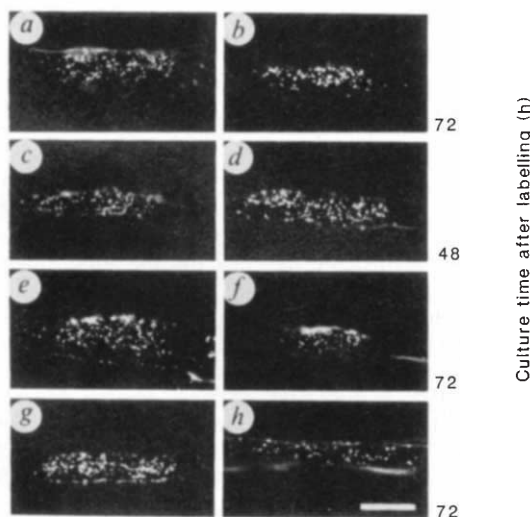


FIG. 1 Autoradiographs of endplates after various treatments affecting the degradation of synaptic AChRs in chronically denervated muscle. The left-hand column represents endplates that were fixed immediately after labelling and rinsing, the right-hand those of contralateral muscles of the same experiments, which were treated identically to those on the left except that after labelling they were cultured for the times indicated. a, b, Stimulated muscles; c, d, unstimulated muscles, 24 h of A23187; e, f, muscles stimulated in the presence of $1 \mu\text{M}$ (+)-PN200-110; g, h, control muscles (unstimulated, no pharmacological treatment). Scale bar, $20 \mu\text{m}$.

METHODS. Soleus muscles of male Sprague-Dawley rats (about 100 g) were denervated bilaterally by sectioning the sciatic nerve under nembutal anaesthesia (1 ml kg^{-1}). The muscles were excised 14 to 26 days later and, after being transferred to organ culture, were chronically stimulated in 100-Hz trains of 1 s duration, applied once every 100 s and/or given one of various pharmacological treatments (Table 1). Stimulus pulses were of alternating polarity, 6 mA strong and 2 ms long except for the experiments on muscle stimulation in the presence of $10 \mu\text{M}$ TTX where the corresponding

pulse parameters were 15 mA and 4 ms. In each experiment, at least two pairs of contralateral soleus muscles from the same animals were treated identically. They were then incubated together with untreated (but denervated) control muscles for 2–4 h in a solution containing $0.2\text{--}0.6 \mu\text{g ml}^{-1}$ [^{125}I] α -bungarotoxin (Amersham; specific activity $> 200 \text{ Ci mmol}^{-1}$) and extensively rinsed. Of each muscle pair, one was then immediately fixed in 2.5% glutaraldehyde in PBS (pH 7.4) whereas the other was transferred to organ culture for up to 77 h before fixation as described previously⁴. Co-culturing of test and control muscles in the same culture dish allowed exclusion of the possibility of changes in AChR half-lives because of culture conditions. After culture, muscle viability was ascertained by visual examination of twitch contraction on electrical stimulation. For autoradiography, the muscles from each experiment were processed simultaneously as described⁴. Autoradiograms were developed before the highest grain density in the non-cultured muscles (0 h cultivation time) had reached $0.5 \mu\text{m}^{-2}$, and photographed on a Zeiss Standard microscope with dark field illumination at $\times 1,000$. Even beyond this range of grain densities, the response of the emulsion is linear⁴. To determine AChR stabilities, densities of autoradiographic grains at junctional and extrajunctional membranes were counted at $\times 1,000$ magnification in dark-field illumination, using the ocular grid of the microscope, and background densities were subtracted. The average grain density determined in a muscle after a certain cultivation time was normalized to the average grain density counted in the contralateral non-cultured muscle of the same pair—the density at 0 h cultivation time was set to 100%—so that the data from different sets of experiments using the same experimental protocol but different cultivation times could be compared. Metabolic stability of the labelled AChRs was determined from the rate of loss of the specific relative endplate grain density after various times of cultivation. All data points (N) were fitted by the Levenberg-Marquart method, assuming first order kinetics for AChR degradation even though at some synapses AChR degradation deviated from single exponential (see Fig. 2b). The calculated values for AChR half-lives are, therefore, only approximations, and, thus, were termed apparent half-lives, $t_{1/2,\text{app}}$. Possible explanations are the presence of a heterogeneous AChR population with different stabilities in the endplate membrane²⁴ or the use of muscles with differing denervation times before treatments, resulting in slightly different junctional AChR stabilities at the onset of the experiments. The calculated values for $t_{1/2,\text{app}}$ were corrected for α -bungarotoxin unbinding according to $1/t_{1/2,\text{app}}(\text{degrad}) = 1/t_{1/2,\text{app}}(\text{measured}) - 1/t_{1/2}(\text{unbinding})$ (ref. 25) with $t_{1/2}(\text{unbinding}) = 36 \text{ days}^{26}$.

TABLE 1 Effects of various physiological and pharmacological treatments on apparent half-lives ($t_{1/2,app}$) of synaptic and extrasynaptic AChRs

Protocol	Days denervated	$t_{1/2,app}$ synaptic (days)	N	$t_{1/2,app}$ extra-synaptic (days)	N
Control	14–15	2.8	24	1.0	63
	20–26	2.5	75	1.0	149
Stimulated	12 h	11.1*	42	1.1	64
	24 h	13.1*	67	1.1	23
A23187 (0.5 μ M)	12 h	6.8*	26	0.7	52
	24 h	14.4*	85	1.2	244
Ryanodine (0.3 μ M)	24 h	3.1	45	1.0	61
	48 h	3.1	20	1.2	64
Stimulated +D600 (10 μ M)	12 h	2.3	56	0.9	82
	24 h	2.6	9	1.3	22
Stimulated (+)-PN200-110 (1 μ M)	24 h	2.3	70	0.9	211
Stimulated +TTX (10 μ M)	24 h	9.3*	50	1.0	99
(+)-SDZ 202-791 (5 μ M)					
+KCl (15 mM)	24 h	7.6*	51	0.8	55
KCl (15 mM)	24 h	2.5	32	ND	

For each protocol, between two and four pairs of muscles were examined in separate experiments. N is the total number of grain-counted fibres, from which $t_{1/2,app}$ was estimated. Controls for each category of test muscles were processed identically except for stimulation or pharmacological treatment. For details of methods see Figs 1 and 2 legends. To determine α -bungarotoxin binding after ryanodine treatment, muscles were homogenized in 10 mM sodium phosphate buffer (pH 7.4) containing NaCl (150 mM), EDTA (10 mM), PMSF (0.1 mM), and 0.5% Triton X-100 at a ratio 10 ml buffer per g wet tissue. Toxin binding was assayed using the DEAE-filter disk method²³.

* $t_{1/2,app}$ values significantly different ($P < 0.05$, one-tailed t -test) in test and control muscles.

ND, not determined.

AChRs is higher in the latter. The $t_{1/2,app}$ of the junctional AChRs in stimulated muscle was estimated to be 11–13 days, that of junctional AChRs in non-stimulated muscle was about 2.5 days. The effect of muscle activity was specific to junctional AChRs as the half-life of extrajunctional AChRs was about 1 day in both stimulated and non-stimulated muscle.

One way in which muscle activity might be linked to stabilization of junctional AChRs is by the associated increase in cytosolic Ca^{2+} activity. To test for Ca^{2+} involvement, muscles were cultured without stimulation in the presence of the Ca^{2+} ionophore A23187 (0.5 μ M) and tetrodotoxin (TTX; 10 nM) for 24 h before labelling (Fig. 1c and d). The $t_{1/2,app}$ of junctional AChRs in ionophore-treated muscle increased to 14.4 days compared with 2.5 days in untreated controls cocultured in the same dish after ionophore had been washed out (Fig. 2). As with muscle stimulation, the stabilizing effect of ionophore treatment was restricted to the junctional AChRs, the $t_{1/2,app}$ of the extrajunctional AChRs remaining at 1.2 days. This suggests that the decreased turnover of junctional AChRs was not due to a nonspecific toxic effect of the ionophore. Also, the effect of ionophore treatment was not due to an indirect effect on membrane potential, as the resting potentials of muscle fibres after 24 h of drug treatment were not significantly different from

those of untreated control fibres (untreated: 63.3 ± 0.8 mV, $N = 30$; treated: 62.0 ± 0.7 mV, $N = 30$).

Activity-dependent AChR stabilization is not mediated by Ca^{2+} released from the sarcoplasmic reticulum (SR). Muscles were cultured without stimulation for 24–48 h in the presence of ryanodine (0.3 μ M), a plant alkaloid known to release Ca^{2+} from the SR^{5–7}, and of TTX (10 nM). Ryanodine did not affect the metabolic stability of junctional AChRs, their $t_{1/2,app}$ remaining low (3.1 days). As an independent test of the effectiveness of ryanodine in releasing Ca^{2+} from the SR, its effect on the number of AChRs in chronically denervated muscle⁸ was examined: 24–48 h of ryanodine treatment at the same concentration reduced [¹²⁵I] α -bungarotoxin binding to 40–60% ($N = 3$) of that in contralateral untreated control muscles, probably as a result of the release of Ca^{2+} suppressing AChR messenger RNAs⁹. This suggests that intracellular Ca^{2+} activity had, indeed, been increased under our culture conditions.

During muscle activity Ca^{2+} may enter the muscle fibre through voltage-activated membrane channels. To test for a role of Ca^{2+} influx in endplate AChR stabilization we examined the effect of blocking Ca^{2+} influx on receptor half-lives in stimulated muscle. To test the possibility of Ca^{2+} entering the muscle fibre through the 'TTX-resistant' Na^{+} channels which appear after denervation¹⁰, we stimulated the muscles, through platinum plate electrodes placed above and below the endplate region, in the presence of 10 μ M TTX, which is sufficient to block such channels¹¹. Currents causing muscle contractions in the stimulated region produced a significant increase in $t_{1/2,app}$ to 9.3 days. Thus, AChR stabilization is not dependent on Ca^{2+} entry through TTX-resistant Na^{+} channels.

Another way for Ca^{2+} to enter the fibre during muscle activity is through voltage-dependent Ca^{2+} channels. In innervated adult skeletal muscle, only one type of Ca^{2+} current, I_{slow} , has been observed. It is slowly activated and inactivated by depolarization and is blocked by dihydropyridines such as (+)-PN200-110 or by the verapamil derivative D-600^{12–14}. To test whether activity-dependent AChR stabilization was mediated by I_{slow} we examined the half-lives of receptors in muscle stimulated in the presence of 1 μ M (+)-PN200-110. Indeed, this drug did block the AChR-stabilizing effect of muscle stimulation (Fig. 1e and f), the apparent $t_{1/2,app}$ of endplate AChRs averaging 2.3 days. The effect was not specific for the dihydropyridine as 10 μ M D-600 also prevented AChR stabilization by stimulation ($t_{1/2,app} = 2.3$ days). Conversely, culturing the muscles in medium containing 15 mM K^{+} in the presence of the Ca^{2+} channel activator (+)-SDZ 202-791 (5 μ M)¹⁵, which prolongs the open state of Ca^{2+} channels¹⁶, increased $t_{1/2,app}$ of the junctional AChRs to 7.6 days, whereas 15 mM K^{+} alone left $t_{1/2,app}$ unaffected at 2.5 days. Neither treatment with (+)-PN200-110 nor with D-600 abolished muscle contractions, indicating that Ca^{2+} continued to be released from the SR in the presence of these drugs. In addition to ryanodine's lack of effect, this is independent evidence that AChR stability is not affected by Ca^{2+} released from the SR. Thus, activity-dependent AChR stabilization in denervated muscle is mediated by I_{slow} .

The resting membrane potential of the cultured muscle fibres

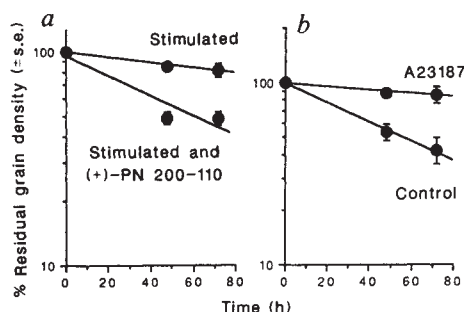


FIG. 2 Degradation of junctional AChR-[¹²⁵I] α -bungarotoxin complex as a function of time, as determined by quantitative autoradiography at endplates of chronically denervated muscles. a, Degradation after stimulation *in vitro* for 24 h with ($t_{1/2,app} = 2.3$ days) and without (+)-PN-200-110 ($t_{1/2,app} = 13.1$ days); b, degradation after treatment with 0.5 μ M Ca^{2+} ionophore A23187 for 24 h compared with untreated muscles, each in the presence of 10 nM TTX. Apparent half-lives, $t_{1/2,app}$, were 14.4 and 2.5 days respectively. Each data point represents the mean $\pm$ s.e. of $N = 6$ –43. No error bars are shown where symbols are larger than s.e.

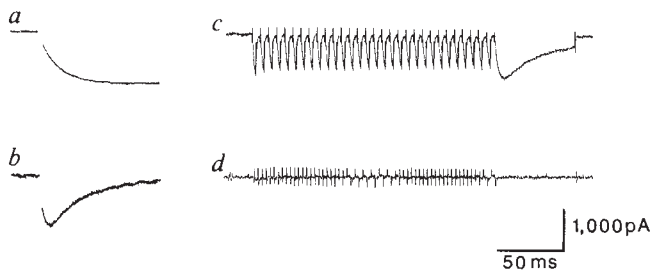


FIG. 3 Activation of dihydropyridine-sensitive Ca^{2+} currents (I_{slow}) in response to depolarizations to +20 mV in fibres dissociated from flexor digitorum brevis muscle of 3–8-day neonatal rats. Holding potentials were –60 mV. *a*, During steady depolarization at 21 °C; *b*, during steady depolarization at 36 °C; *c*, during repetitive 5 ms depolarizations followed by steady depolarization, 36 °C; *d*, during repetitive 5 ms depolarizations, 36 °C, in the presence of 1 μM (+)-PN200-110; *b*, *c* and *d* are from the same cell.

METHODS. Flexor digitorum brevis muscles were dissociated essentially as described in ref. 13 and plated onto collagen-coated culture dishes. They were allowed to settle for 3–4 h in MEM supplemented by 10% FCS. For electrophysiological experiments, the medium was replaced by a solution containing (in mM): CaCl_2 (10), tetraethyl ammonium chloride (145), HEPES (10), buffered to pH 7.2 with CsOH. Ca^{2+} currents were measured conventionally by the whole cell clamp technique. The pipette solution consisted of (mM): CsCl (140), MgCl_2 (5), EGTA (10), HEPES (10), buffered with CsOH to pH 7.2. Linear components of capacitive transients and leak currents were subtracted after digital scaling of control currents elicited upon hyperpolarizations by 20 mV. The remaining transients were retouched.

was about –60 mV. The available data as to whether I_{slow} at this potential can be activated at all^{12,17} are conflicting, which makes a role of this current in Ca^{2+} -dependent AChR stabilization questionable. Therefore, we have re-examined the voltage dependence of I_{slow} in freshly dissociated fibres from flexor digitorum brevis muscles of neonatal rats. When elicited from holding potentials of –80 and then from –60 mV by voltage jumps to +20 mV, the amplitude of I_{slow} at 20–22 °C was little affected ($I_{\text{slow},60}/I_{\text{slow},80} = 0.86 \pm 0.07$, $N = 10$). To examine whether the slow activation of I_{slow} allows an influx of Ca^{2+} during brief depolarizations such as action potentials, we mimicked a 100-Hz train of action potentials by repetitive depolarizations from –60 mV to +20 mV, each 5 ms in duration. At 20–22 °C, activation of I_{slow} in most cells was too slow to be resolved in the capacitive current transients. Raising the bath temperature to 36 °C increased the rate of Ca^{2+} channel activation sufficiently (Fig. 3*a, b*) to elicit significant inward currents (Fig. 3*c*) of 200–800 pA ($N = 7$). This was between 30 and 70% of the maximum current during a longer depolarization at the end of the train. These findings are, therefore, consistent with a role of I_{slow} in mediating AChR stabilization.

In chronically denervated muscle, another type of Ca^{2+} channel might be expressed which is fast-activating and in normal muscle development disappears within 3 weeks of birth¹⁸. Even if such channels had been present, however, their current I_{fast} could not have been involved in AChR stabilization, because (1) it was largely inactivated by holding the cells for >60 s at –60 mV ($I_{\text{fast},60}/I_{\text{fast},80} = 0.12 \pm 0.06$, $N = 9$), and (2) it was not blocked by (+)-PN200-110 (which inhibits AChR stabilization in stimulated muscle) even after 3 h exposure.

In summary, our experiments show that metabolic stabilization of synaptic AChRs in denervated muscle can be induced in organ culture by less than a single day of electrical stimulation and is then maintained for at least 3 days. Activity-dependent AChR stabilization is specific for endplate receptors and is

mediated by an influx of Ca^{2+} through dihydropyridine-sensitive channels; Ca^{2+} released from the SR is ineffective, suggesting that it is rapidly sequestered before it reaches the muscle fibre membrane, possibly by the numerous mitochondria present in the endplate region of the myoplasm.

The dihydropyridine-sensitive Ca^{2+} channels are thought to be located in the T-tubules of the muscle fibre where they have a dual role as voltage sensors in excitation–contraction coupling¹⁹ and in the replenishment of the SR with Ca^{2+} . Unless muscle fibres express another, hitherto unknown type of dihydropyridine-sensitive Ca^{2+} channel, our experiments demonstrate a new role for these channels in endplate development and maintenance. The fact that they mediate stabilization of endplate AChRs, coupled with the restricted diffusion of Ca^{2+} in the cell, suggests that they may also occur in the endplate membrane.

In denervated muscle, the number of dihydropyridine-sensitive Ca^{2+} channels is increased²⁰ which may increase the activity-dependent Ca^{2+} influx and thus support the stabilization of AChRs. During endplate formation, on the other hand, when innervation is intact and the number of Ca^{2+} channels is low¹⁸, AChR stabilization may also depend on the Ca^{2+} influx through AChR channels²¹.

The mechanism by which Ca^{2+} influx leads to AChR stabilization is unknown. One hypothesis is that they may be protected from degradation by binding to hypothetical anchoring sites specific to the endplate membrane¹: AChRs might bind to these sites in a Ca^{2+} -dependent fashion and thus become stabilized. Both these sites and the way in which Ca^{2+} affects AChR binding to them remain to be elucidated.

The significance of activity-induced, Ca^{2+} -dependent AChR stabilization for endplate function may be to compensate for activity-induced, Ca^{2+} -dependent reduction in AChR synthesis^{9,22}, thus allowing maintenance of synaptic AChR accumulation in normally active muscle. □

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Suppression of tumorigenicity in human colon carcinoma cells by introduction of normal chromosome 5 or 18

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DEVELOPMENT of colon carcinomas can be associated with allelic deletions on several chromosomes, including 5q and 18q (refs 1–10). The APC gene^{11–13} on 5q and the DCC gene¹⁴ on 18q have been identified as potential tumour suppressor genes, whose suppression contributes to colon carcinogenesis. To investigate the role of genes in these deleted regions, we have now introduced a single normal human chromosome into a human colon carcinoma cell line, COKFu, through microcell hybridization^{15–20}. Several clones of hybrid cells containing normal chromosome 5, and others containing normal chromosome 18, were obtained. The morphology of the hybrid cells was markedly altered: the hybrids with chromosome 5 exhibited a closely packed polygonal morphology, and the hybrid cells with chromosome 18 were flattened. The cloning efficiency of the hybrid cells in soft agar was reduced from 0.46 to 0% of that of the parental carcinoma cells, and the tumorigenicity of these hybrid cells in athymic nude mice was completely suppressed. The growth properties of the hybrid cells with chromosome 11 were not substantially changed. These results strongly suggest that the genes on normal chromosome 5 and 18 function as tumour suppressors in colon carcinogenesis.

A colon carcinoma cell line, COKFu, was established from a poorly differentiated sporadic adenocarcinoma. COKFu cells were fused with the microcells formed from mouse A9 cells containing a single normal human chromosome 5, 11, or 18 (ref. 21), by the microcell hybridization method described in the legend to Table 1. Introduced chromosomes were tagged by a neomycin-resistance gene. The fused cells were cultured in normal medium for 2–4 days, and then in medium containing the neomycin analogue G418 (400 $\mu\text{g ml}^{-1}$). G418-resistant colonies of COKFu-microcell hybrid (hybrid cell) were formed after 2–3 weeks culture, and flat colonies were purified by further cultivation in the presence of G418.

Morphology of the hybrid cells is shown in Fig. 1. The parental COKFu cells were spindle-shaped, whereas the hybrid cells containing normal chromosome 5 showed the closely packed

polygonal morphology typical of epithelial cells, and the hybrid cells containing normal chromosome 18 had a flattened shape, different from that induced by chromosome 5. Hybrid cells containing normal chromosome 11 resembled parental cells. The modal chromosome number of COKFu was 92, and those of the hybrid cells with normal chromosomes 5, 18, and 11 were either 93 or 94. Introduction of each exogenous chromosome into COKFu cells after microcell hybridization was proved by restriction fragment length polymorphism markers in Southern hybridization (Fig. 2), as it was difficult in karyotypic analysis to ascertain that the additional chromosomes were introduced into the tetraploid cells. The presence of a tagged normal chromosome in the hybrid cells was confirmed by hybridization with pSV2neo as probe. Each DNA from the hybrid cells containing chromosome 5, 18, or 11 showed the same band as that in DNA of the corresponding A9 clone from which the normal human chromosome was derived. Moreover, hybridization of D5S6 (5q11.2–q13.3) to DNA from the hybrid cells containing normal chromosome 5, that of D18S5 (18q21.3–qter) to DNA from the hybrid cells containing normal chromosome 18, or that of *int2* (11p13) to DNA from the hybrid cells containing normal chromosome 11 revealed the presence of an additional allele on chromosome 5, 18, or 11. Several G418-resistant clones without additional allele of D5S6 or D18S5 were obtained after introduction of normal chromosome 5 or 18. Although being positive for hybridization with pSV2neo, these clones had a morphology similar to that of the parental COKFu and formed tumours in nude mice.

The characteristics of the parental COKFu and its microcell hybrids are summarized in Table 1. Doubling time of the hybrid cells with normal chromosome 5 or 18 in liquid medium was 2–3 times that of the parental cells. The cloning efficiency of the hybrid cells with normal chromosome 5 and that of cells with normal chromosome 18 in soft agar were reduced to 0.46–0.05% and 0.02–0%, respectively. Introduction of normal chromosome 11 did not significantly alter the growth properties of COKFu in both liquid medium and soft agar. Tumorigenicity was assayed by inoculation of the cells into athymic nu/nu mice. The parental COKFu cells formed palpable tumours in all mice within 1 week of subcutaneous injection of 5×10^6 – 10^7 cells, and the tumours developed to 250 mm³ over 4 weeks. However, neither hybrid cells with normal chromosome 5 nor those with normal chromosome 18 developed tumours, even over a period of 6 months after inoculation. The hybrid cells containing normal chromosome 11 formed tumours in all mice, although the growth rate of the tumours was reduced to about half that of the parental cell tumours.

These results suggest that suppression of tumour phenotypes in the hybrid cells was brought about by normal tumour suppressor genes on normal chromosome 5 and 18 introduced into the colon carcinoma cells. The parental COKFu showed allele

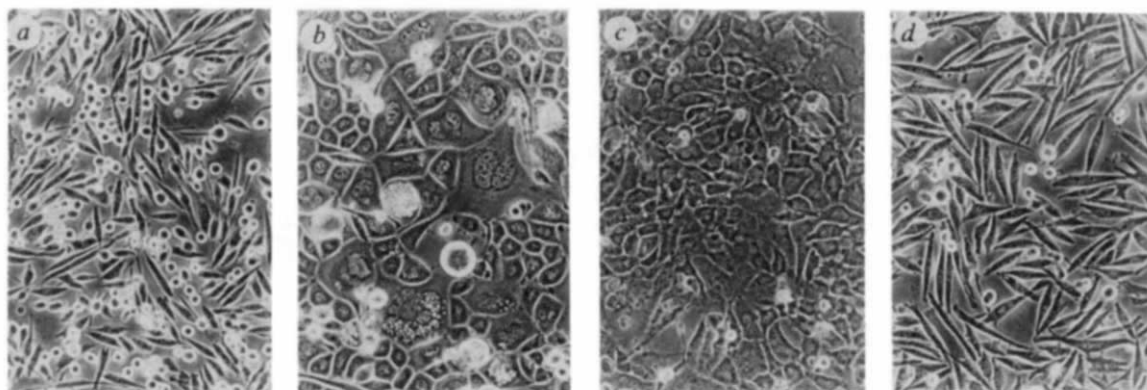


FIG. 1 Phase-contrast photomicrographs of colon carcinoma cells COKFu and COKFu-microcell hybrids ($\times 90$). a, COKFu, the parental colon carcinoma

cell line; b, Fu(Ch5)-3; c, Fu(Ch18)-1; d, Fu(Ch11)-2; b–d are COKFu-microcell hybrid cell lines containing normal chromosome 5, 18, or 11 respectively.

TABLE 1 Growth characteristics of COKFu-microcell hybrids

Cell line	Growth in G418	Modal chromosome number	Doubling time (h)	Cloning efficiency in agar*	Tumorigenicity†
COKFu	—	92	17.8	1,635±193 (100)	4/4
COKFu(Ch5)-3	+	93	44.5	7.5±2.8 (0.46)	0/4
COKFu(Ch5)-6	+	94	30.2	0.8±0.5 (0.05)	0/3
COKFu(Ch18)-1	+	94	38.0	0	0/3
COKFu(Ch18)-15	+	93	49.3	0.3±0.2 (0.02)	0/2
COKFu(Ch11)-2	+	93	18.6	885±139 (54.1)	3/3
COKFu(Ch11)-5	+	93	20.4	881±143 (53.1)	3/3

Microcell-mediated chromosome transfer was performed as described²¹. Mouse A9 cells containing a single human chromosome 5, 11 or 18 tagged with a neomycin-resistance gene (*neo*) were plated at a density of 10^6 cells per 25 cm² flask, and 2 days later, cells were treated for 48 h with colcemid ($0.02 \mu\text{g ml}^{-1}$) to form micronuclei. Microcells were collected by centrifugation in serum-free medium containing cytochalasin B ($10 \mu\text{g ml}^{-1}$). Microcell pellets were resuspended into serum-free medium and filtered successively through 8- μm , 5- μm , and 3- μm polycarbonate filters, to remove large microcells, contaminating whole cells, and karyoplasts. Purified microcells were incubated with 6×10^5 – 8×10^5 COKFu cells for 20 min in the presence of phytohaemagglutinin P ($50 \mu\text{g ml}^{-1}$), and then treated with polyethylene glycol followed by washing in serum-free medium. After 2–4 days cultivation in DMEM containing 15% FBS, COKFu cells were split into three dishes (100 mm) containing G418 ($400 \mu\text{g ml}^{-1}$) and were cultivated for 2–3 weeks. G418-resistant colonies were isolated from dishes and purified further. Five independent microcell-mediated introductions of normal chromosome 5 were done. Several G418-resistant clones with morphological alteration were obtained in two experiments, including COKFu(Ch5)-1 from experiment No. 1 and COKFu(Ch5)-3 from No. 3. Six independent introductions of chromosome 18 were done; COKFu(Ch18)-1 was obtained from experiment No. 3 and COKFu(Ch18)-15 from No. 6. For analysis of chromosome numbers, cells were treated with colcemid ($0.1 \mu\text{g ml}^{-1}$) followed by treatment with 0.075 M KCl and fixation in methanol:glacial acetic acid (3:1). Air-dried chromosome preparations were stained with Giemsa, and 100 metaphases per hybrid clone were analysed. To determine the growth rate, cells were plated in 35-mm plastic petri dishes at a density of 4×10^4 cells with DMEM containing 15% FBS and cultivated for 7 days. Every other day, the cells in two dishes were counted. To assay colony formation in soft agarose, 10^4 cells in 0.3% agarose medium (2.5 ml) containing 5% FBS were layered onto 0.5% agarose medium (5 ml) containing 5% FBS in a 60-mm dish. After incubation for 2 weeks, the number of colonies ($>135 \mu\text{m}$ in diameter) was counted. The values are means of four plates. Tumorigenicity was tested by subcutaneous injection of 5×10^6 – 10^7 tumour cells in 4–6 week-old athymic BALB/c nu/nu mice. All animals were observed for tumour formation for up to 3–6 months after injection. COKFu: parental colon carcinoma cell line; Fu(Ch5), Fu(Ch18), Fu(Ch11): COKFu-microcell hybrid cell containing normal human chromosome 5, 18, or 11.

* Number of colonies per 10^4 cells (%).

† Number of animals with tumour/number of animals inoculated.

loss on chromosome 18, but not on chromosome 5, by the use of D5S37 (5q21–22), which was the only informative probe. In the previous investigation allele loss on 5q was observed in 50–60% of colon carcinomas^{3,9}, and on 18q in 50–80% of advanced carcinomas^{6,10}, suggesting that inactivation of tumour suppressor genes on chromosome 5 and 18 is common in advanced colon carcinomas. Recessiveness of the APC gene on 5q (ref. 3) and that of the DCC gene on 18q (ref. 14) have also been demonstrated. Accordingly, both the APC gene on 5q and the tumour suppressor gene (or DCC gene) on 18q in COKFu were probably inactivated, and the inactivation was released by the introduced chromosomes.

It has been reported that the tumorigenic phenotype was

re-expressed when the introduced chromosome was lost from the microcell hybrids^{15,16,18}. Such revertants have been formed in slowly growing tumours in nude mice or have arisen during the cultivation of hybrids in back-selection medium, possibly due to instability of the introduced chromosome. However, neither microcell hybrids of COKFu with chromosome 5 nor those with chromosome 18 developed tumours in nude mice, even 6 months after being inoculated. Cultivation *in vitro* of these hybrid cells in the absence of G418 also failed to generate G418-sensitive revertants. These results suggest that the introduced chromosome 5 or 18 in COKFu hybrid cells is rather stable compared with other chromosomes in cases previously reported. It is unlikely that G418-resistant flat cells, possibly

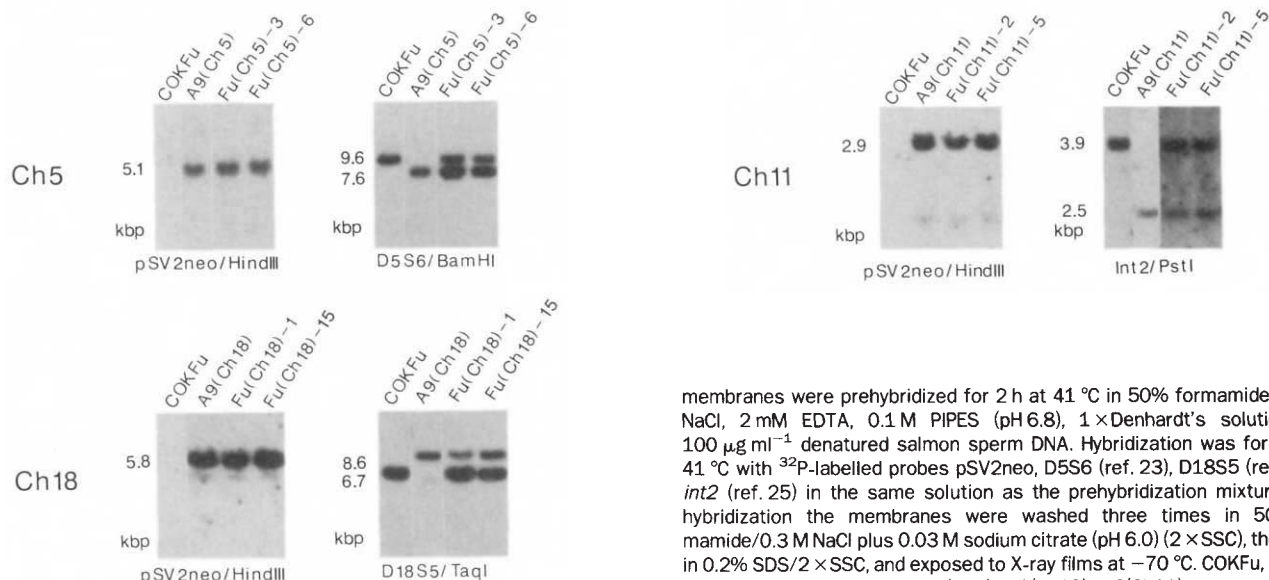


FIG. 2 Southern blot hybridization²² of DNA from COKFu and COKFu-microcell hybrids containing normal chromosome 5, 18 or 11 was performed. The

membranes were prehybridized for 2 h at 41 °C in 50% formamide, 0.75 M NaCl, 2 mM EDTA, 0.1 M PIPES (pH 6.8), 1×Denhardt's solution, and $100 \mu\text{g ml}^{-1}$ denatured salmon sperm DNA. Hybridization was for 20 h at 41 °C with ³²P-labelled probes pSV2neo, D5S6 (ref. 23), D18S5 (ref. 24) or *int2* (ref. 25) in the same solution as the prehybridization mixture. After hybridization the membranes were washed three times in 50% formamide/0.3 M NaCl plus 0.03 M sodium citrate (pH 6.0) (2×SSC), then twice in 0.2% SDS/2×SSC, and exposed to X-ray films at –70 °C. COKFu, parental colon carcinoma cell line; A9(Ch5), A9(Ch18), A9(Ch11), mouse A9 cells containing normal human chromosome 5, 18, or 11; Fu(Ch5), Fu(Ch18), Fu(Ch11), COKFu-microcell hybrid cell line containing normal human chromosome 5, 18, or 11. kbp, kilobase-pairs.

pre-existing in parental COKFu, could have been selected by chance during cultivation with G418, as no G418-resistant cell was present among at least 2×10^7 parental COKFu cells. Moreover, morphological change was undoubtedly caused by introduced chromosomes, as the morphology of the hybrid cells was distinctive, depending on the kind of chromosome introduced. Independent clones of hybrid cells with chromosome 5, Fu(Ch5)-3 and Fu(Ch5)-6 from different experiments, had a similar epithelial-cell-like shape, and independent hybrid clones containing chromosome 18 all had a similar flattened shape, as shown in Fig. 1. Such chromosome-specific morphologies may reflect the different functions of tumour suppressor genes on chromosomes 5 and 18.

Suppression of tumorigenicity by a single chromosome has been observed in several tumours. Introduction of a single copy of normal fibroblast chromosome 11 into HeLa cells (near-tetraploid) was sufficient to suppress tumorigenicity¹⁶, and a single normal chromosome 11 suppressed the tumorigenic expression of Wilms' tumour cells¹⁵. Although we cannot show the exact number of normal chromosomes introduced into each hybrid clone, a single copy of these chromosomes may also suppress the malignant phenotypes if recessiveness (complete inactivation) of tumour suppressor genes on chromosome 5 and 18 is essential for the malignant growth of COKFu.

In advanced colon carcinomas, several tumour suppressor genes on various chromosomes, including 5q, 17p, 18q, and/or 22q, are assumed to be inactivated simultaneously. Our present data suggest that if one of them is supplied on a normal chromosome, it causes a decrease in the malignancy of colon carcinoma cells and suppresses certain malignant phenotypes, such as morphology, growth in soft agar, and tumorigenicity in nude mice, although not all tumour phenotypes are suppressed. Hybrid cells containing chromosome 5 or 18 grew more rapidly than epithelial cells from normal mucosa, and were still immortal *in vitro*. Simultaneous introduction of several different chromosomes may cause stronger suppression of carcinoma cell growth. The mechanism of suppression of tumorigenicity by a normal chromosome is not yet understood. Introduction of a normal chromosome into carcinoma cells may bring about diverse biochemical alterations with a decrease in the malignancy. Preliminary data on northern hybridization of poly(A)⁺ RNA from hybrid cells indicated that introduction of chromosome 5 into COKFu cells resulted in decreased expression of *myc* and *p53* genes, and increased expression of the *K-ras-2* gene without significant change in transcription of the *H-ras-1* gene. Expression of the DCC gene in messenger RNA was hardly

detectable in parental COKFu cells when an amplified fragment of 233 base pairs (bp) (nucleotides 986–1,218 in DCC complementary DNA (ref. 14)) was hybridized to synthetic 57-mer oligodeoxynucleotide (1,141–1,197 in DCC cDNA). After introduction of normal chromosome 18, considerable amounts of the 233-bp fragment were detected in hybrid clones COKFu(Ch18)-1 and COKFu(Ch18)-15 (Fig. 3), although the level of transcription was lower than in normal mucosa. Introduction of normal chromosome 5 or 11 did not affect expression of the DCC gene. Alterations in the expression of other cellular oncogenes and cancer-related genes or markers should be investigated further in these microcell hybrids. □

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Cloning by functional expression of platelet-activating factor receptor from guinea-pig lung

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PLATELET-activating factor (PAF), a unique phospholipid mediator, possesses potent proinflammatory, smooth-muscle contractile and hypotensive activities, and appears to be crucial in the pathogenesis of bronchial asthma and in the lethality of endotoxin and anaphylactic shock^{1–3}. Despite this, little is known of the molecular properties of the PAF receptor and related signal transduction systems. Although several lines of evidence suggest that activation of the PAF receptor stimulates phospholipase C and subsequent inositol trisphosphate formation through G protein(s)^{4,5}, the PAF receptor and calcium channel are reported to show a close relation^{2,6}. As a first approach to cloning lipid autacoid

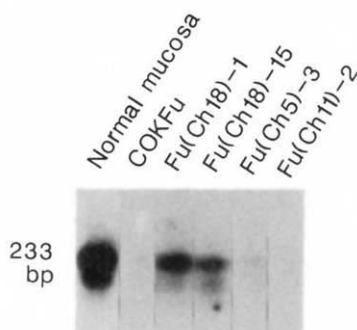


FIG. 3 Expression of DCC gene in COKFu and COKFu-microcell hybrids containing normal chromosome 5, 18, or 11. Poly(A)⁺ RNA was extracted from COKFu, COKFu-microcell hybrids and normal mucosa. First strand cDNA was synthesized using antisense primer 5'-AGCCTCATTTTCAGCCACACA-3', and then the 233-bp fragment was amplified by the PCR method using the antisense primer and sense primer 5'-TTCGCCATGGTTTAAATCA-3' as described by Fearon *et al.*¹⁴. The PCR products were separated through agarose gel, transferred to nitrocellulose membrane, and hybridized to ³²P-labelled synthetic 57-mer oligodeoxynucleotide containing the sequence 1,141–1,197 in DCC cDNA. The names of samples are the same as those in Table 1 and Fig. 2.

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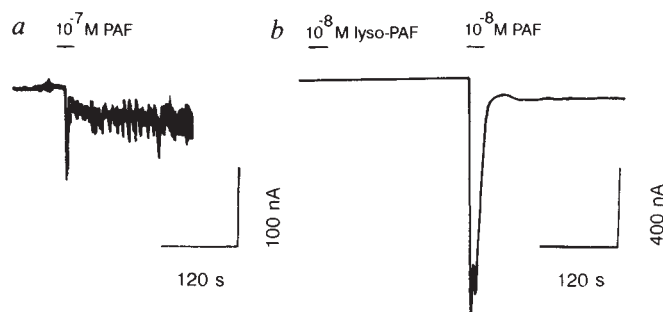


FIG. 1 PAF-induced inward Cl^- current in *Xenopus* oocytes. *a*, The electrophysiological response to PAF in an oocyte injected with the transcript made of a 6×10^4 -phage DNA library. *b*, The electrophysiological response to

lyso-PAF and PAF in an oocyte injected with the transcript of the cloned PAF receptor cDNA. Lyso-PAF did not elicit a response; PAF induced a large inward current.

METHODS. *a*, DNA was extracted from a 6×10^4 λ -Zap II phage pool, digested with *NotI* and transcribed *in vitro* by T7 RNA polymerase (Stratagene) in the presence of cap analogue 7-methyl GpppG (New England Biolabs). The transcript, dissolved in Milli Q^R water, was injected into defolliculated oocytes (about 50 ng in 50 nl per oocyte) and incubated at 20 °C for 2–3 days. The oocyte membrane potential was maintained at -60 to -100 mV in modified Ringer's solution (115 mM NaCl, 2 mM KCl, 1.8 mM CaCl_2 , 5 mM HEPES, pH 7.4) containing 0.1% BSA, and the Cl^- current elicited by bath-applied PAF was recorded. *b*, Transcript (~ 5 ng) of plasmid Zp74 digested with *NotI* was injected into oocytes. Electrophysiological recording was performed as above. The reversal potential for the response to PAF is -28 ± 2.2 mV (mean $\pm$ s.d., $n=3$), indicating that the depolarizing response is mainly due to opening of Cl^- channels.

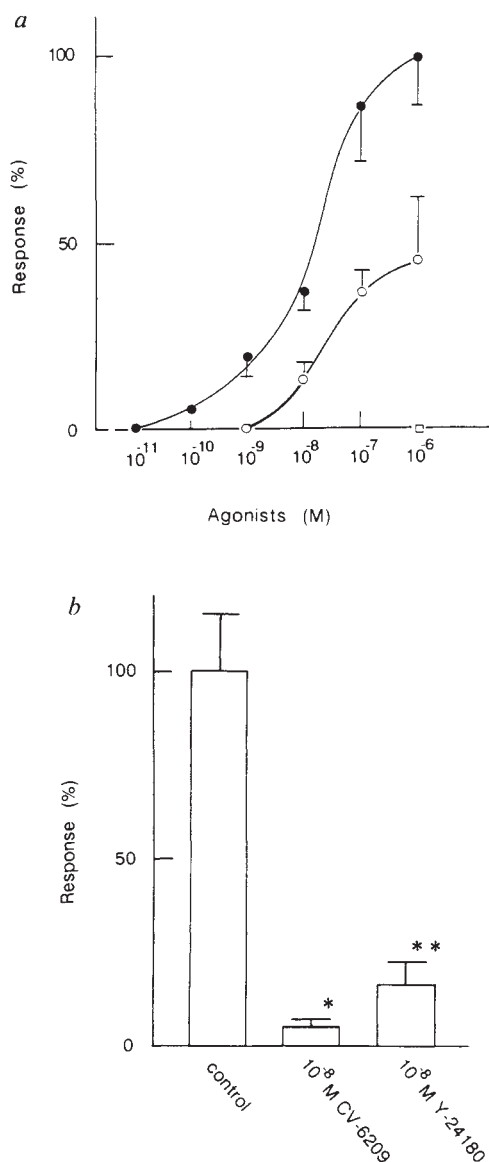


FIG. 2 Pharmacological properties of the protein encoded by the cloned cDNA expressed in oocytes and COS-7 cells. *a*, Dose-response curve of PAF (closed circle), 1-O-octadecyl-2-acetamido-2-deoxy-glycero-3-phosphocholine (compound II)¹⁰ (open circle) and lyso-PAF (open square) in eliciting electrophysiological response in oocytes injected with the transcript of cloned plasmid Zp74. The mean amplitude of the current induced

by 10^{-8} M PAF was defined as 100% ($n=9$, 1,110 nA). Other points, means of 5 oocytes; vertical bars, s.e.m. PAF elicited inward current in a concentration-dependent manner with an ED_{50} around 10 nM. Compound II induced smaller responses than PAF, and lyso-PAF at the concentration of $1 \mu\text{M}$ did not elicit an inward current. *b*, Inhibition of PAF-induced response by specific antagonists CV-6209 and Y-24180. Data expressed as the percentage of the mean current amplitude of oocytes not treated with the antagonists (1,100 nA). Vertical columns and bars, mean and s.e.m., respectively ($n=4$). Data with and without the antagonists were tested for differences of means by Student's *t*-test. * and **, $P < 0.01$. *c*, Binding characteristics of [3H]WEB-2086 to membrane from COS-7 cells expressing PAF receptor encoded by the cloned cDNA. Saturation isotherm of specific (open circle) and non-specific (closed circle) bindings and the derived Scatchard plot (inset) are presented. Each point represents the mean of duplicate determinations. Results are shown from one of three independent experiments. In this experiment, K_d and B_{max} values were 6.4 nM and 6.9 pmol mg^{-1} protein, respectively.

METHODS. *a*, Oocytes were injected with ~ 5 ng of the transcript of Zp74 in 50 nl water. After incubation for 2 days at 20 °C, oocytes were voltage-clamped at -80 to -100 mV and inward currents elicited by bath-applied PAF (closed circle), compound II (open circle) and lyso-PAF (open square) were recorded. *b*, After voltage clamp of the oocytes at -80 to -100 mV, 10^{-8} M CV-6209 or Y-24180 was bath-applied for 2 min. Then, 10^{-8} M PAF was bath-applied in the presence of the antagonists for 30 sec and PAF-induced currents were recorded. *c*, The full-length PAF receptor cDNA was subcloned into the eukaryotic expression vector, pEUK-Cl (Clontech). This plasmid was transfected to COS-7 cells by the DEAE-dextran method²¹. After a 3-day culture, cells were collected by scraping and homogenized in 25 mM HEPES, pH 7.4 containing 10 mM MgCl_2 and 0.25 M sucrose. Preparation of the membrane fraction and ligand binding assay were performed as described previously¹⁴. Nonspecific binding was determined in the presence of $10 \mu\text{M}$ unlabelled PAF.

receptors, we have isolated complementary DNA for the PAF receptors. Our strategy involved gene expression in *Xenopus laevis* oocytes and electrophysiological detection of PAF-induced responses. Sequence analysis indicates that the receptor belongs to the superfamily of G protein-coupled receptors.

Purification of the PAF receptor has thus far been unsuccessful, and PAF differs in structure from the ligands for previously cloned receptors. We therefore decided to use a ligand-specific cloning strategy using the gene expression system in *Xenopus laevis* oocytes⁷ instead of the usual biochemical or homology probing approaches. A cDNA library was constructed (Fig. 1) from size-fractionated guinea-pig lung poly(A)⁺ RNA which elicited electrophysiological responses to PAF when injected in *Xenopus* oocytes (between 18S and 28S ribosomal RNA, see Fig. 3b). To screen the library, transcript was made *in vitro* using DNA of 6×10^4 phage clones as a template, and the transcript was injected into *Xenopus* oocytes. After 2–3 days incubation, the oocytes were tested for electrophysiological responses to PAF. As a small but discrete response was obtained (Fig. 1a), we subdivided the phage clones by monitoring ability to induce PAF responsiveness, and isolated a functional phage clone designated Z74. Z74 was converted to a plasmid Zp74 by a rescue excision protocol, and the transcript was again shown to induce PAF-dependent response in oocytes (Fig. 1b).

The protein encoded by the insert of Zp74 was identified as the PAF receptor based on its pharmacological properties when expressed in oocytes and COS-7 cells. As shown in Fig. 2a, oocytes showed dose-dependent increases in the amplitude of the response with the expected sensitivity to PAF (half maximal response around 10 nM)^{8,9}. A partial agonist of PAF, 1-O-octadecyl-2-acetamido-2-deoxyglycero-3-phosphocholine¹⁰, induced smaller responses than those elicited by PAF. Lyso-PAF did not induce a response even at the concentration of 1 μ M (Fig. 2a). An antagonistic PAF analogue CV-6209 (ref. 11) and a structurally different antagonist, Y-24180 (ref. 12), inhibited PAF-induced responses at the expected potencies (Fig. 2b). To determine the ligand-binding properties of the encoded receptor, the full-length cDNA was subcloned into the mammalian expression vector pEUK-CI (Clontech) and transiently expressed in COS-7 cells. Membranes prepared from

COS-7 cells transfected with the expression plasmid specifically bound a PAF antagonist, WEB 2086 (ref. 13) in a concentration-dependent manner with a dissociation constant of 6.4 nM (Fig. 2c). This value was comparable to those reported in guinea pig and human lung membranes (16.8 and 22.6 nM, respectively)¹⁴.

Northern blot analysis showed that three discrete bands corresponding to the cloned cDNA were present in different proportions among the tissues (Fig. 3a, arrows). The relative potential of size-fractionated mRNA to induce PAF sensitivity (Fig. 3b) and the size of the cloned cDNA (3,020 base pairs) indicate that transcript 2 is PAF-receptor mRNA. We cannot determine whether the other transcripts (1 and 3) come from different post-transcriptional modifications of the PAF-receptor transcript or from other cross-hybridizing RNAs. Transcript 2 was abundant in leukocytes with smaller amounts in spleen, lung and kidney (Fig. 3a). This distribution is consistent with the fact that PAF modulates the functions of mesangial cells¹⁵ as well as leukocytes and platelets.

Figure 4a shows a 3,020-nucleotide sequence of the PAF receptor cDNA and the 342-amino-acid sequence (calculated relative molecular mass, 38,982) deduced from the longest open reading frame of the cloned DNA. An in-frame tripeptide (double underlined) is found 30 bases upstream of the putative initiation codon. Such a small open reading frame in the 5' flanking sequence has been found in hamster β_2 -adrenergic receptor and rat D₂-dopamine receptor cDNAs^{16,17}. Hydropathy profile analysis revealed the existence of seven hydrophobic, putative transmembrane segments characteristic of G protein-coupled receptors. As shown in an alignment with some members of the family (Fig. 4b), several amino acids are highly conserved; these include an aspartic acid in the second transmembrane segment, two cysteines in the second and third extracellular loops which may make a disulphide bond, and three prolines in the sixth and the seventh transmembrane segments which may contribute to the ligand-binding pocket¹⁸. Although PAF contains a positively-charged choline moiety, the aspartic acid in the third helix, a putative counter ion for cationic ligands¹⁸ (catecholamines and acetylcholine), is not present. The cytoplasmic tail of the PAF receptor contains four serines and five threonines as possible phosphate acceptors.

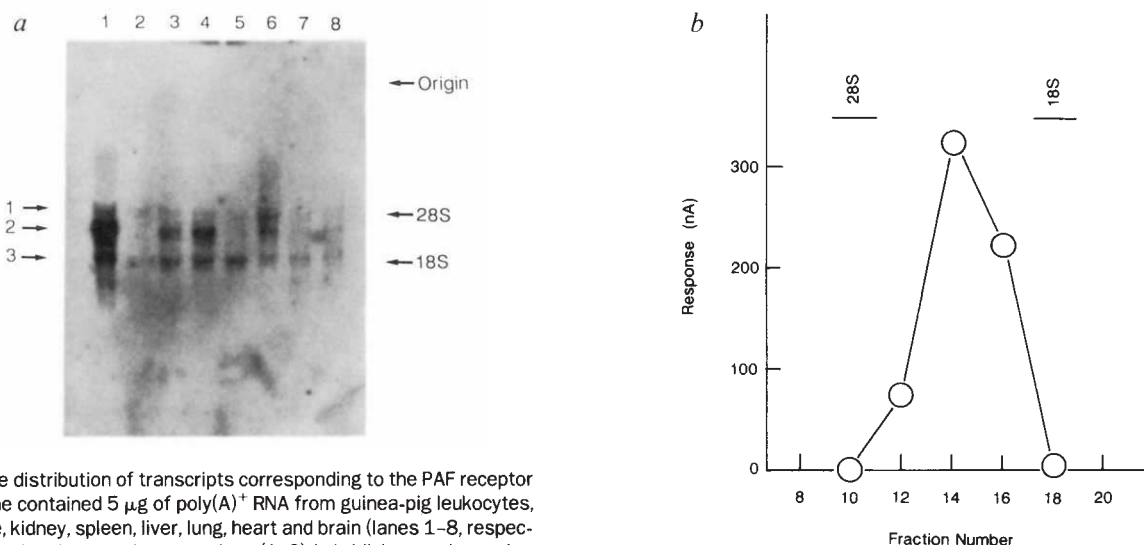


FIG. 3 a, Tissue distribution of transcripts corresponding to the PAF receptor clone. Each lane contained 5 μ g of poly(A)⁺ RNA from guinea-pig leukocytes, small intestine, kidney, spleen, liver, lung, heart and brain (lanes 1–8, respectively). Arrows, the three main transcripts (1–3) hybridizing to the probe. Size markers, guinea pig rRNA. b, Relative abilities of size-fractionated mRNA to induce PAF-responsiveness in oocytes. Smaller fraction number indicates larger RNA. 18S and 28S, positions of 18S and 28S rRNA, respectively. METHODS. a, Poly(A)⁺ RNA was prepared from guinea-pig tissues, resolved on a 0.7% agarose gel containing 20% formaldehyde (5 μ g per lane) and blotted onto a Hybond-N⁺ nylon membrane (Amersham). The blot was hybridized with ³²P-labelled Zp74 insert at 65 °C for 16 h. The filter was successively washed at 65 °C in 3 $\times$ SSC, 0.1% SDS for 20 min, 2 $\times$ SSC,

0.1% SDS for 20 min and finally 0.1 $\times$ SSC, 0.1% SDS for 10 min before being exposed for 6 d to an X-ray film at –70 °C with an intensifying screen. b, Poly(A)⁺ RNA was size-fractionated by ultracentrifugation on a continuous sucrose density gradient (5–30%) and separated into 30 fractions. The size was determined by agarose gel electrophoresis and aliquots of each fraction were tested for the potential to evoke PAF-responsiveness in oocytes as described in Fig. 1.

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Control of the sperm–oocyte switch in *Caenorhabditis elegans* hermaphrodites by the *fem-3* 3' untranslated region

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IN the *Caenorhabditis elegans* hermaphrodite germ line, sperm and then oocytes are made from a common pool of germ-cell precursors. The decision to differentiate as a sperm or an oocyte is regulated by the sex-determining gene, *fem-3*. Expression of *fem-3* in the hermaphrodite germ line directs spermatogenesis and must be negatively regulated to allow the switch to oogenesis^{1,2}. In adult hermaphrodites (which are producing oocytes), most *fem-3* RNA is found in the germ line³, consistent with both the requirement for *fem-3* in hermaphrodite spermatogenesis and the maternal effects of *fem-3* on embryonic sex determination^{1,2}. Whereas loss-of-function mutants in *fem-3* produce only oocytes, hermaphrodites carrying any of nine *fem-3* gain-of-function (*gf*) mutations make none; instead sperm are produced continuously and in vast excess over wild-type amounts¹. Genetic analyses suggest that *fem-3(gf)* mutations have escaped a negative control required for the switch to oogenesis¹. Here we report that all nine *fem-3(gf)* mutants carry sequence alterations in the *fem-3* 3' untranslated region (3' UTR). There is no increase in the steady-state level of *fem-3(gf)* RNA over wild-type, but there is an increase in the polyadenylation of *fem-3(gf)* RNA that is coincident with the unregulated *fem-3* activity. Results of a titration experiment support the hypothesis that a regulatory factor may bind the *fem-3* 3' UTR. We speculate that *fem-3* RNA is regulated through its 3' UTR by binding a factor that inhibits translation, and discuss the idea that this control may be part of a more general regulation of maternal RNAs.

To learn how *fem-3(gf)* mutations affect the regulation of *fem-3*, we sequenced two *fem-3(gf)* alleles, *q20* and *q22*, in their entirety and found in each case only a single base pair (bp) change in the 3' UTR (Fig. 1a, b). We then sequenced the 3' UTRs of the remaining seven *fem-3(gf)* mutants and found single-base pair changes in a 5-bp region in six of the alleles and a 112-bp deletion in the seventh and strongest allele (Fig. 1b, c). The finding of mutations in all nine *fem-3(gf)* 3' UTRs suggests this is the sole defect responsible for the *fem-3(gf)* phenotype. We propose that the switch from sperm to oocytes is regulated by the wild-type *fem-3* 3' UTR.

How does the 3' UTR regulate *fem-3* activity? One possibility is that it affects RNA levels. In *fem-3(gf)* mutants, *fem-3* might be activated by an increase in the synthesis or stability of *fem-3* RNA. To investigate this possibility, we compared wild-type and *fem-3(gf)* steady-state RNA levels (Fig. 2a, b). In the third and fourth larval stages (L3 and L4), the level of *fem-3* RNA in *fem-3(gf)* animals is similar or slightly less than that of wild

type (Fig. 2a, b), whereas in *fem-3(gf)* adults it is markedly decreased (Fig. 2b). The decrease seen in *fem-3(gf)* adults probably results from a lack of oocytes and not from the *gf* mutation itself: *fem-3* RNA levels are no longer reduced in a *fem-3(gf)* mutant making oocytes (*q20q77*, a *fem-3* gene carrying both *gf* and loss-of-function mutations in *cis*; ref. 1) (Fig. 2b). These results indicate that *fem-3(gf)* mutations probably do not act by increasing the steady-state levels of *fem-3* RNA and, further, that they are likely to act post-transcriptionally.

Comparison of *fem-3(+)* and *fem-3(gf)* RNAs also revealed an increase in the size and heterogeneity of *fem-3(gf)* RNAs in L4 and adult animals (Fig. 2c, and data not shown) that is not observed in mid-L3 to mid-L4 RNAs (Fig. 2a). To test whether this change was due to increased polyadenylation of *fem-3(gf)* RNAs, we compared RNAs with and without their poly(A) tails. In mid- to late-L4 animals, *fem-3(q96gf)* RNA migrates in a broader band than wild type (Fig. 2c, lanes 1 and 2), but when poly(A) tails are removed, the RNAs comigrate (Fig. 2c, lanes 3 and 4). Similar results were obtained for L4 and adult RNAs of both *fem-3(q20gf)* and *fem-3(q96gf)* (data not shown). We estimate that *fem-3(gf)* poly(A) tails range from 30 to 100 A residues, whereas *fem-3(+)* poly(A) tails are of ~30–50 residues (data not shown, see legend to Fig. 2). The developmental stages at which increased polyadenylation of *fem-3(gf)* RNA is observed coincide with the stages at which *fem-3(gf)* is inappropriately activated to make sperm.

One simple interpretation of the data is that *fem-3(gf)* mutations destroy a binding site for a negative regulator of *fem-3* RNA activity (Fig. 3a). Unable to bind the negative regulator, *fem-3(gf)* RNA would remain active and cause continuous

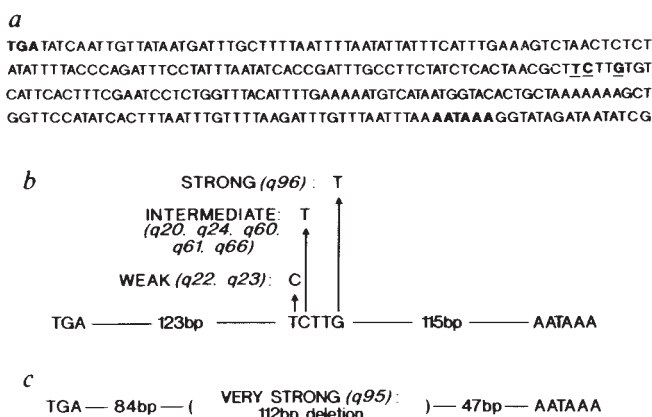


FIG. 1 Sequences of wild-type and mutant *fem-3* 3' UTRs: **a**, Wild-type *fem-3* 3' UTR sequence starting with base 1,402; numbering based on complementary DNA sequence and analysis of gene structure (T. Rosenquist, J.A. and J.K., manuscript in preparation). The translational stop and polyadenylation signal are in bold; nucleotides altered by *fem-3(gf)* point mutations are bold and underlined. No striking secondary structure (for example, stem-loop) is obvious near the mutations, and no sequences complementary to the *fem-3* 3' UTR are found in any other part of *fem-3*. **b**, *fem-3(gf)* point mutations. Mutants of equivalent strength¹ carry identical changes. **c**, *fem-3(q95gf)* deletion. The junction sequence created by the deletion (CTGCTGGT) is identical in 6 out of 8 positions to the wild-type region altered by *fem-3(gf)* point mutations (CTTCTGT). Also, *fem-3(q95gf)* contains a G to A change at nucleotide 1,652, the G following the polyadenylation signal. METHODS. For each allele, a genomic library was made by cloning *Bgl*II-digested homozygous mutant DNA into the *Bam*HI site of λ EMBL3. Phage containing *fem-3* (as a 10 kb *Bgl*II fragment) was purified; a 5.5-kb *Sall*-*Bgl*II fragment containing the entire transcribed region of *fem-3* was subcloned from the λ phage into pIB176. Dideoxy chain termination sequencing was performed by standard methods using Sequenase (USB) and primers to *fem-3*. Both *q20gf* and *q22gf* were sequenced in the entire transcribed region plus 100 bp of flanking DNA; the other alleles were sequenced only in the 3' UTR.

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spermatogenesis (Fig. 3b). If this hypothesis is true, exogenously expressed wild-type 3' UTR RNA would titrate this factor from endogenous *fem-3* RNA, whereas *fem-3(gf)* 3' UTR RNA should lack or have reduced titrating activity (Fig. 3c, d). Titration of a negative regulator is predicted to activate endogenous *fem-3* RNA inappropriately, leading to masculinization of the hermaphrodite germ line. To test this idea, we constructed plasmids designed to express either a wild-type (p-*wt*) or *fem-3(gf)* (p-*q95gf* or p-*q96gf*) 3' UTR. In each case, the *fem-3* 3' UTR and its 3' flanking region were placed downstream of the *fem-3* 5' flanking region and 50 nucleotides of the *fem-3* 5' UTR. The plasmids, which lacked the *fem-3* coding region, were introduced into wild-type animals (see legend to Fig. 4). As predicted, we found that p-*wt* masculinized the hermaphrodite germ line (Fig. 4e, f), but that p-*q95gf* or p-*q96gf* had little masculinizing activity (Fig. 4d, f). We interpret this result to mean that exogenous *fem-3(+)* 3' UTR, but not *fem-3(gf)* 3' UTR, competes with the endogenous wild-type *fem-3* 3' UTR for a negative regulator. The masculinizing activity is likely to be due to synthesis of 3' UTR RNA, because a plasmid containing only the wild-type 3' UTR plus 3' flanking DNA (p-*wt* 3' only) or one containing *fem-3* 5' flanking plus 5' UTR sequences

alone (p-*wt* 5' only) does not masculinize wild-type animals (Fig. 4f and legend to Fig. 4; T. Evans, personal communication).

Our working model is that late in L4 the wild-type *fem-3* 3' UTR binds a negative regulatory factor, which leads to shortening of the poly(A) tail and inactivation of *fem-3* RNA. Inhibition of *fem-3* would effect the switch to oogenesis (Fig. 3a). In other species, the lengthening of poly(A) tails on maternal RNAs can cause an increase in their translation⁴⁻⁷; we speculate that *fem-3(gf)* RNAs have escaped a negative translational control. The regulation could occur through a direct interaction with the translation or polyadenylation machinery, or be an indirect effect, for example, by localization of *fem-3* RNA to a compartment where it cannot be translated. There are precedents for the involvement of the 3' UTR in regulating translation⁴⁻¹⁰, and in the case of creatine kinase B regulation, soluble factor(s) have been shown to bind the 3' UTR *in vitro*¹¹.

How does negative regulation through the *fem-3* 3' UTR relate to the mechanism of sex determination in *C. elegans*? Gain-of-function mutations in *tra-2*, which interfere with a negative regulation required for the onset of hermaphrodite spermatogenesis^{12,13}, likewise fall in the 3' UTR of *tra-2* RNA (P. G. Okkema and J.K., manuscript in preparation). Because *fem-3(gf)* and *tra-2(gf)* mutations primarily affect the hermaphrodite germ line^{1,12,13}, this regulation probably does not operate in all tissues for the determination of sex in *C. elegans*. Instead, the proposed translational regulation of these genes through their 3' UTRs may control spermatogenesis in hermaphrodites. As *fem-3* is a maternal RNA (T. Rosenquist, J.A. and J.K., manuscript in preparation), we speculate that the regulation of *fem-3* by its 3' UTR may be related to a general mechanism of 'masking' maternal RNAs. Perhaps, in the evolution of hermaphroditism, transient spermatogenesis has been achieved by using an existing germ-line control of maternal messages. Masking

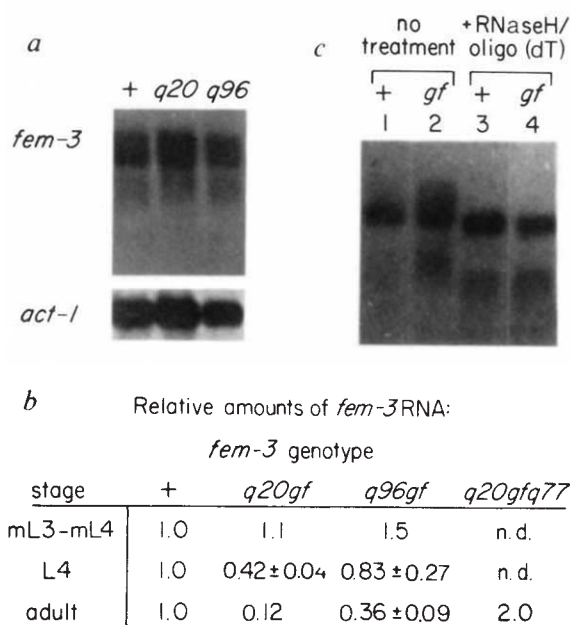


FIG. 2 Analysis of *fem-3(gf)* RNAs. **a**, Northern blot of mid-L3 to mid-L4 RNA prepared from *fem-3(+)*, *fem-3(q20gf)* and *fem-3(q96gf)* animals. Top panel, *fem-3* RNA; bottom panel, *act-1* RNA. **b**, Relative amounts of *fem-3* RNA in *fem-3(+)* and *fem-3* mutant animals. Numbers indicate the *fem-3* to *act-1* ratio, with wild type being set at 1.0. The variance shown for some entries gives the range of results from experiments done twice. L3, third larval stage; L4, fourth larval stage. **c**, Northern blot of wild-type (lanes 1 and 3) and *fem-3(q96gf)* (lanes 2 and 4) mid-late L4 RNAs before and after removal of poly(A) tails. Lanes 1 and 2, before treatment; lanes 3 and 4, RNAs treated with oligo(dT) and RNase H to remove poly(A) tails. **METHODS.** Embryos were isolated using hypochlorite treatment of gravid adults grown at 15 °C, the permissive temperature for *fem-3(gf)* (ref. 1), transferred to 25 °C, the restrictive temperature for *fem-3(gf)*, and incubated until they reached the desired stage. Both wild-type and *fem-3(gf)* strains carry the linked marker *dpy-20(e1282)*. For northern blots, 1–5 µg poly(A)⁺ RNA were used per lane; preparation of poly(A)⁺ RNA, formaldehyde-agarose gel electrophoresis, blotting and hybridization were as described³; *fem-3* and *act-1* were detected using ³²P-labelled RNAs made from pJK50, a partial *fem-3* cDNA in pIB176 and pT3/T7-18-103, an *act-1* specific clone (provided by M. Krause), respectively. Signals on northern blots were quantitated on a Betagen β scanner. RNase H digestion of poly(A) tails was essentially as described¹⁴. Poly(A) tail lengths were measured on northern blots by comparing the size of *fem-3* to markers of known size.

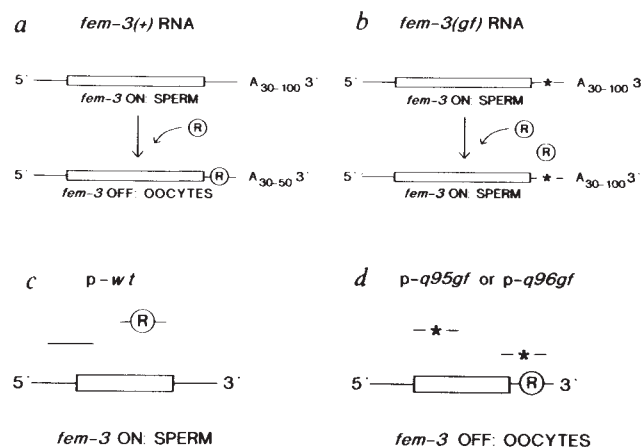


FIG. 3 Model for *fem-3* regulation and experimental design to test the model: *fem-3* RNA is represented by the open box (denoting coding region) with lines (5' and 3' UTRs); the star denotes a *fem-3(gf)* mutation. R, negative regulator. **a** and **b**, Model. In **a**, *fem-3* RNA is active when no regulator is bound to its 3' UTR and its poly(A) tail is relatively long; *fem-3* RNA is inactivated by binding a regulator to its 3' UTR and shortening its poly(A) tail. Active *fem-3* RNA directs spermatogenesis, whereas inactivation of *fem-3* RNA leads to the switch from spermatogenesis to oogenesis. In **b**, *fem-3(gf)* RNA is unable to bind the negative regulator; its poly(A) tail remains long and the RNA active resulting in continuous spermatogenesis. **c** and **d**, Test of the model. Construction of plasmids (p-*wt*, p-*q95gf* and p-*q96gf*) described in legend to Fig. 4. **c**, The exogenous expression of wild-type *fem-3* 3' UTR (solid lines) by p-*wt* is predicted to titrate R from endogenous *fem-3* RNA, causing inappropriate activity of *fem-3* and production of sperm instead of oocytes. **d**, The exogenous expression of a *fem-3(gf)* 3' UTR (line with star) by p-*q95gf* or p-*q96gf* is predicted not to titrate R from endogenous *fem-3* RNA, resulting in little or no effect on *fem-3* activity and production of oocytes in adults as usual.

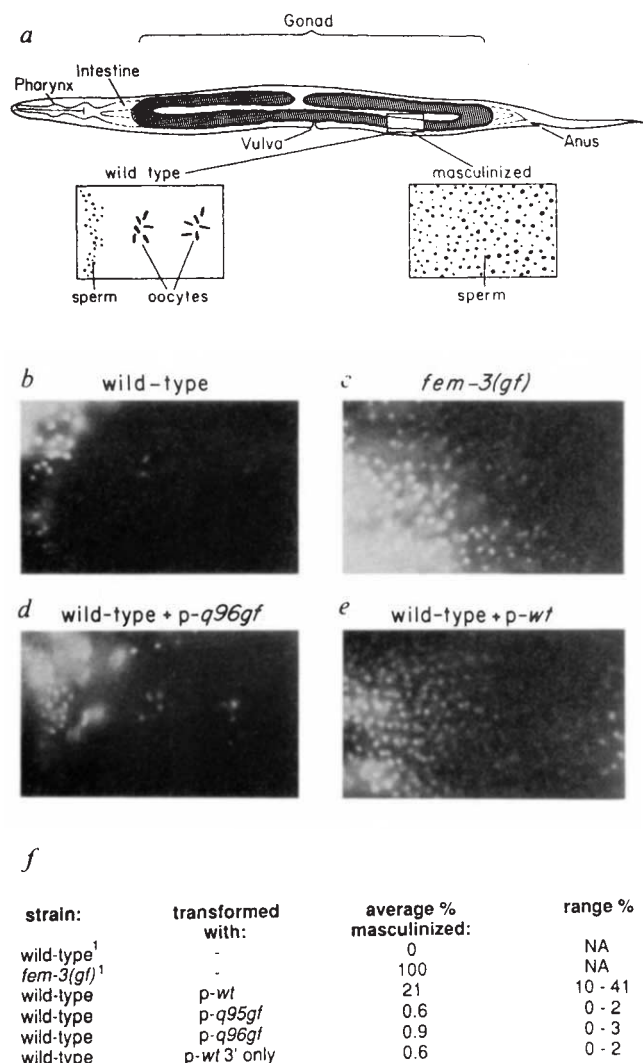


FIG. 4 Exogenous *fem-3(+)* 3' UTR masculinizes the germ line. *a*, Anatomy of a *C. elegans* adult hermaphrodite. Gonad is shaded; boxed region contains differentiated gametes and is enlarged to show a representation of DNA staining patterns from wild-type or masculinized germ lines. Dots represent condensed sperm nuclei; clusters of short bars represent oocyte chromosomes in meiotic diplotene. *b-e*, Photomicrographs of gametes from DAPI-stained worms. *b*, Untransformed wild-type (sperm plus two oocytes); *c*, untransformed *fem-3(q20gf)* (sperm only); *d*, wild-type transformed with p-q96gf (sperm plus two oocytes); *e*, wild-type transformed with p-wt (sperm only). *f*, Percentage of wild-type, *fem-3(gf)* and transformed animals with a masculinized germ line. The average percentage masculinization includes data from several independent lines. Number of lines and data: six p-wt lines (10%, 11%, 21%, 25%, 41%); three p-q95gf lines (0%, 0%, 2%); three p-q96gf lines (0%, 0%, 3%); three p-wt 3' only lines (0%, 0%, 1%). METHODS. Plasmids were constructed using a plasmid carrying 1.2 kb of 5' flanking region and 50 bp of 5' UTR from *fem-3* (p-wt 5' only, pJK228; T. Evans, personal communication); the backbone is pPD16.43 (ref. 15). After removal of the *lacZ* sequence, a *Clal-HindIII* fragment carrying the entire 3' UTR plus 755 bp of 3' flanking region from *fem-3(+)*, *fem-3(q95gf)* or *fem-3(q96gf)* was inserted to make p-wt (pJK275), p-q95gf (pJK277) or p-q96gf (pJK276), respectively; p-wt 3' only (pJK222) is this wild-type *Clal-HindIII* fragment inserted into pPD16.43. Transformation was as described¹⁶, except that injection was into distal gonadal arms. Wild-type animals were coinjected with 100 $\mu\text{g ml}^{-1}$ of the plasmid of interest plus 100 $\mu\text{g ml}^{-1}$ pRF4, which contains a dominant *rol-6* gene. pRF4 incorporated into extrachromosomal arrays causes animals to roll (C. Mello, J. Kramer, V. Ambros and D. Stinchcomb, personal communication). Rollers contained from 10 to 50 copies of *fem-3* DNA of the appropriate size as determined by Southern blot quantitation, normalizing for the percentage of rollers (data not shown). For determination of percentage masculinization, L2 to L3 rollers were picked, grown to adulthood (2 days at 25 °C), and examined by Nomarski microscopy. An animal was counted as masculinized if at least one gonad arm contained only sperm. From 50 to 150 rollers were scored for each line. Of three p-wt 5' only lines, one was examined by Nomarski microscopy; 0/50 animals were masculinized. The other two lines were examined in the dissecting microscope. Hermaphrodites with a masculinized germline produce neither oocytes nor eggs which is a distinctive phenotype; no such animals were detected (T. Evans, personal communication). For DAPI staining, rollers were washed in distilled H₂O, incubated in methanol or ethanol containing 200 ng ml⁻¹ DAPI for 20 min, washed once in distilled H₂O and mounted on agarose pads for observation and photography (E. Lambie, personal communication).

machinery might become activated during hermaphrodite spermatogenesis and turn off *fem-3*, effecting the switch to oogenesis. Consistent with this idea is the discovery of *mog-1*, a candidate negative regulator of *fem-3*, which is also maternally required for embryogenesis (P. Graham and J.K., unpublished). Perhaps *mog-1* is involved in masking *fem-3* and other maternal messages; the masking of *fem-3* could turn *fem-3* off, resulting in the switch from spermatogenesis to oogenesis. □

MAP2 kinase and 70K S6 kinase lie on distinct signalling pathways

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ACTIVATION of protein synthesis is required for quiescent cells to transit the cell cycle¹, and seems to be mediated in part by phosphorylation of the 40S ribosomal protein, S6 (ref. 2). A mitogen-activated S6 kinase of relative molecular mass 70,000 (70K) has been isolated from mouse fibroblasts^{3,4} as well as from avian, rat and rabbit tissues⁵⁻⁹. Comparison of complementary DNA sequences shows that this enzyme¹⁰ is distinct from S6 kinase II (92K)¹¹ found in *Xenopus* eggs¹² and fibroblasts^{13,14}. Both kinases are activated by serine/threonine phosphorylation^{3,15-18}, suggesting that at least one serine/threonine kinase links receptor tyrosine kinases with S6 kinases. A candidate for this link is MAP2 kinase, which is rapidly activated by tyrosine/threonine phosphorylation following mitogenic stimulation¹⁹⁻²³. Incubation of MAP2 kinase from insulin-treated 3T3-L1 adipocytes with phosphatase-inactivated S6 kinase II from *Xenopus* leads to partial reactivation and phosphorylation of the enzyme¹⁷. These and other findings⁸ have led to the suggestion that MAP2 kinase also activates the

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70K S6 kinase. Here we refute this idea by showing that the two kinases lie on distinct signalling pathways.

Treatment of quiescent fibroblasts with serum or epidermal growth factor (EGF) causes a transient increase in MAP2 kinase activity (Fig. 1a). Maximal activation of 3-fold (serum) and 3.4-fold (EGF) was observed at 2.5 min. S6 kinase was activated to a higher level but at a slower rate, increasing 6.8- and 9.3-fold after 10 min with serum or EGF, respectively (Fig. 1b). The EGF-stimulated MAP2 kinase seems to be identical to the enzyme from insulin-treated 3T3-L1 adipocytes^{17,22,24} on the basis of its chromatographic behaviour (see Fig. 3b), immunoprecipitation with anti-phosphotyrosine antibodies, reaction on western blots with a polyclonal antibody against starfish MAP2 kinase, and inactivation by treatment with phosphatase 2A (data not shown).

The ability of MAP2 kinase to reactivate 70K S6 kinase was tested using control S6 kinase that was not exposed to phosphatase and an aliquot that was 92% inactivated by treatment with phosphatase 2A (Fig. 2a). The enzymes were incubated with or without MAP2 kinase in the presence of Mg-ATP and a

phosphatase inhibitor. The results of our initial experiments were difficult to interpret because the S6 kinase loses most of its activity during the incubation. MAP2 kinase prevents this decrease, resulting in apparent 'activation' of the control S6 kinase and 'reactivation' of the phosphatase-treated enzyme. This effect is due to the presence of stabilizing components in the MAP2 kinase storage buffer and is eliminated by including ethylene glycol, Triton X-100 and albumin in the reactivation mixture. Under these conditions, the control and phosphatase-treated S6 kinases were stable when incubated in the absence of MAP2 kinase (Fig. 2a). When MAP2 kinase was added, the control S6 kinase was not affected and the phosphatase-treated enzyme was not reactivated (Fig. 2a). Similar results were obtained using: (1) five times more MAP2 kinase; (2) S6 kinase purified from rat liver^{7,9} or quiescent fibroblasts¹⁶; (3) MAP2

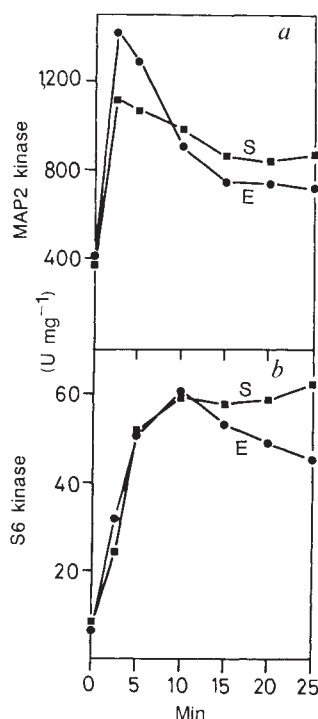


FIG. 1 Activation of *a*, MAP2 kinase and *b*, S6 kinase in cells treated with 10% fetal calf serum (■) or 5 nM EGF (●). Averaged values from three experiments are shown.

METHODS. Quiescent Swiss mouse 3T3 fibroblasts¹⁵ in 10 cm plates were treated for the times indicated and then washed twice with 10 ml 2 °C buffer A (ref. 19). Cells were scraped into 0.4 ml buffer A and extract supernatants were prepared¹⁹, diluted 1:20 in assay buffer (buffer A; ref. 15) and assayed immediately. MAP2 kinase assays contained 5 µl enzyme (0.1 µg protein) and 5 µl assay buffer containing 100 µM [γ -³²P]ATP (10 c.p.m. fmol⁻¹), 0.2 mg ml⁻¹ MAP2, and enough protein kinase inhibitor²⁸ to inhibit 14 U ml⁻¹ cAMP-dependent protein kinase. After 10 min at 37 °C the reactions were stopped and the samples subjected to electrophoresis on 5% acrylamide gels and scintillation counting¹⁵. The assay was linear in terms of time and protein. MAP2 kinase activities measured according to ref. 19 were up to 25 times lower, due to the lower dilution of extract used and inhibition by 2-glycerol phosphate²⁴. S6 kinase activity and protein concentration were assayed as described previously²⁸. One unit of kinase incorporates 1 pmol of P_i into S6 or MAP2 per min. MAP2 was purified to homogeneity from rat brain (B. Brugg and A. Matus, unpublished). The tissue was homogenized, centrifuged at 100,000 *g*, and the supernatant was adjusted to 750 mM NaCl and 2 mM DTT. After boiling for 5 min, the solution was centrifuged again and the supernatant concentrated by ultrafiltration before chromatography on Sepharose 4BCL.

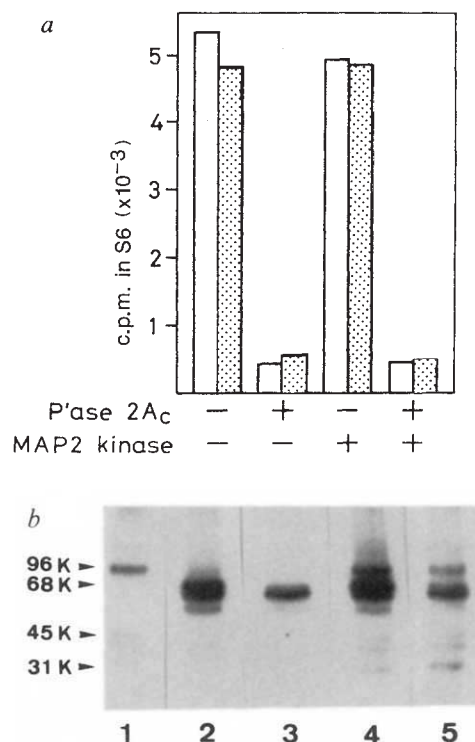


FIG. 2 MAP2 kinase does not activate or phosphorylate S6 kinase. *a*, Effect of MAP2 kinase on S6 kinase activity. S6 kinase was treated without (control; -) or with (+) phosphatase 2A (P'ase 2A_c). The samples were kept on ice (open bars) or at 37 °C (shaded bars) without (-) or with (+) MAP2 kinase and S6 kinase activity assayed. *b*, Phosphorylation of S6 kinase by MAP2 kinase. Enzymes were incubated with [γ -³²P]ATP in the following combinations: lane 1, MAP2 kinase; lane 2, control S6 kinase; lane 3, phosphatase-treated S6 kinase; lane 4, 1 plus 2; lane 5, 1 plus 3.

METHODS. *a*, S6 kinase purified from serum-stimulated cells¹⁶ was incubated with or without 5 U ml⁻¹ of the catalytic subunit of phosphatase 2A (refs 15, 28) for 10 min at 37 °C in 50 mM Tris, 0.1 mM EGTA, 5% ethylene glycol, 5 mM DTT, 10 mM MgCl₂, 0.1% Triton X-100 and 0.25 mg ml⁻¹ BSA, pH 7.5. The samples were diluted 8-fold with buffer containing 10 mM *p*-nitrophenylphosphate (pNPP) and 100 µM ATP (final concentrations) and 5 µl aliquots were kept for 15 min either on ice or at 37 °C with or without 31 U ml⁻¹ of MAP2 kinase. Under these conditions the phosphatase was 97% inhibited. S6 kinase activity was then assayed for 15 min at 37 °C after addition of 5 µl buffer containing 10 mM pNPP, 4 mg ml⁻¹ 40S subunits, protein kinase inhibitor, and [γ -³²P]ATP. MAP2 kinase was partially purified from cells treated for 2.5 min with 5 nM EGF by anion-exchange (see Fig. 3b) and hydrophobic interaction chromatography as described in ref. 24. The activity of the enzyme toward MAP2 was 2,000 times higher than toward S6. Blanks contained either no enzyme or MAP2 kinase alone. *b*, MAP2 kinase, control S6 kinase and phosphatase-inactivated S6 kinase were prepared as described above in buffer without BSA. The enzymes were then incubated for 15 min at 37 °C with 10 mM pNPP and 5 µM [γ -³²P]ATP (350 c.p.m. fmol⁻¹) and subjected to gel electrophoresis¹⁵ and autoradiography.

kinase purified by immunoprecipitation with anti-phosphotyrosine antibodies; and (4) S6 kinase that was only 50% inactivated by phosphatase 2A (data not shown).

In agreement with these results, 70K S6 kinase is not phosphorylated by MAP2 kinase *in vitro*. Incubation of MAP2 kinase with [γ - 32 P]ATP resulted in phosphorylation of a major 96K protein and three minor proteins of 33–47K (Fig. 2b, lane 1). Because MAP2 kinase is a 40K enzyme^{22,24}, the 96K band may be a contaminant and one of the lower bands may represent the enzyme. Autophosphorylated control S6 kinase appears as a doublet at 70K (Fig. 2b, lane 2), probably representing differentially phosphorylated forms of the enzyme¹⁶. After phosphatase inactivation, autophosphorylation is reduced, especially of the upper protein of the doublet (Fig. 2b, lane 3). When MAP2 kinase was incubated together with either active or inactive S6 kinase, no increased phosphorylation of the 70K proteins was detected (Fig. 2b, lanes 4 and 5). The results in Fig. 2 conflict with a recent report claiming that a 70K S6 kinase from rabbit liver is partially reactivated by MAP2 kinase from Rat 1 cells⁸. However, the stability of the S6 kinase used in that study was not measured, nor was phosphorylation of the enzyme by MAP2 kinase shown.

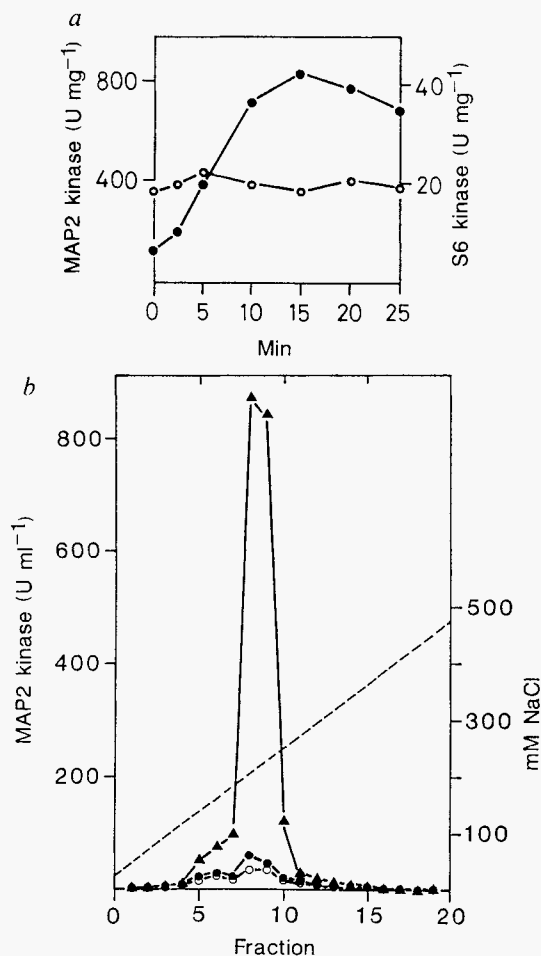


FIG. 3 Effect of insulin on MAP2 kinase activity. *a*, Activation of MAP2 kinase (○) and S6 kinase (●) in cells treated with 1 nM insulin. *b*, Elution of MAP2 kinase during anion-exchange chromatography. ○, Resting cells; ▲, cells treated for 2.5 min with 5 nM EGF; ●, cells treated for 5 min with 1 nM insulin. **METHODS.** *a*, Cells were treated for the times indicated and kinase activities were assayed in extract supernatants as described in Fig. 1. Averaged values from three experiments are shown. *b*, Extract supernatants were prepared from 5–15-cm plates of cells in buffer A (ref. 24). Supernatants were applied to a 1-ml Mono Q column (Pharmacia) at 4 °C, the column was washed with 3 ml buffer A, and the proteins eluted with NaCl (---) at 1 ml min⁻¹. Fractions of 1 ml were collected and diluted 1:20 for assay. Recovery of kinase activity was ≥82%. Most of the activity from resting cells appeared in the flow-through and wash fractions.

The failure of MAP2 kinase to reactivate 70K S6 kinase *in vitro* led us to look at its effect *in vivo*. If MAP2 kinase lies directly upstream of S6 kinase in a phosphorylation cascade, one might expect that the most potent activators of the former enzyme should also be the most potent activators of the latter, but when the effects of ten agents were compared, no such correlation was found (data not shown). One explanation for these results is that some agents may cause activation of more than one MAP2 kinase or of an S6 kinase phosphatase. However, we were surprised to find that insulin was the poorest stimulator of MAP2 kinase among all the agents tested, even though the enzyme was first detected in insulin-treated cells¹⁹. Indeed, insulin caused at best a 1.2-fold increase in MAP2 kinase activity at 5 min, whereas S6 kinase was activated 6.1-fold after 15 min (Fig. 3a). An even more striking difference was seen when extracts were subjected to anion-exchange chromatography. MAP2 kinase activity from insulin-treated cells was barely above resting levels, whereas it was 20 times higher after EGF treatment (Fig. 3b). Even at 5×10^{-7} M insulin, MAP2 kinase activity in extract supernatants never increased more than 1.9-fold, whereas S6 kinase was activated as much as 6.9-fold. Insulin-like growth factor-1 (105 ng ml⁻¹) was also a poor activator of MAP2 kinase, causing a maximal 2.1-fold stimulation after 2.5 min but a 6.1-fold activation of S6 kinase (data not shown).

Together, these data indicate that MAP2 kinase is not the activator of 70K S6 kinase in fibroblasts. The results with insulin (Fig. 3) further imply that activation of the two enzymes is accomplished through different signalling pathways. The 70K S6 kinase kinase remains unidentified. There may be more than one such enzyme, as S6 kinase is activated by a wide range of agents acting through different signalling pathways^{2,25}. The biphasic kinetics of S6 kinase activation induced by EGF (refs 26, 27) could be explained by sequential activation of different S6 kinase kinases acting together to activate the enzyme fully. Finally, preliminary results indicate that the phosphorylation sites which lead to 70K S6 kinase activation are in the C-terminal half of the protein (S. Ferrari, N. Totty and G.T., unpublished data), in a region which has no similarity to S6 kinase II (ref. 10). Experiments designed to identify the S6 kinase activator(s) should show reactivation and phosphorylation at the same S6 kinase sites *in vitro* as observed *in vivo* as well as confirming that the putative S6 kinase kinase responds to mitogens in a consistent manner. □

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Gene therapeutics

P. L. Felgner and G. Rhodes

The direct delivery of purified genes *in vivo* and their application as drugs, without the use of retroviruses, is discussed.

RESEARCHERS from several laboratories have reported recently that functional genes can be administered by direct injection into living animals without the use of retroviruses. In some cases, long-term expression can be obtained *in vivo* without any need to encourage integration of the genes into the host cell genome. The level and persistence of expression obtained in these studies suggests that this research may have scientific and therapeutic applications.

Conceptually, the *in vivo* delivery and expression of recombinant genes for gene therapy, without the use of retroviruses, has several advantages. First, plasmid DNA constructs containing suitable controlling elements are technically easier and less time consuming to prepare and test than retrovirus constructs. Second, manufacturing scale-up is more complex for potentially infectious retrovirus material, than it is for non-infectious, plasmid-derived polynucleotide sequences, which can be grown in conventional fermentation facilities in the same way that recombinant proteins are produced. Although recent advances in retrovirus technology continue to demonstrate that retroviruses can be designed that are, in principle, safe, there is always the concern that use of a virus-based delivery vehicle might lead to deleterious side effects such as virus infection or cancer in a small percentage of patients. These concerns include the possibility of recombination with endogenous viruses, which could potentially mutate into a deleterious infectious form. A retrovirus-based vehicle is likely to induce an immune response against the intrinsic retroviral antigens, rendering repetitive treatments with the same delivery vehicle less effective. And finally, retroviral-mediated gene transfer methods result in stable transformation of target cells. Although this is often regarded as advantageous, the stable transformation of a patient's somatic cells may make it difficult to reverse the treatment regimen if undesirable side effects dictate that it should be stopped. For all these reasons, it is appropriate to consider developing *in vivo* gene transfer techniques that do not use viruses and are reversible.

Among the earliest reported *in vivo* transfections using cloned genes were experiments showing that virus replication could occur following injection of infectious viral genomes *in vivo*¹⁻⁶ (see Table).

LITERATURE SHOWING EXPRESSION FOLLOWING DIRECT INJECTION OF GENES *IN VIVO*

Gene	Vehicle	Tissue	Ref.
<i>Infectious and/or oncogenic cloned DNA</i>			
Polyoma virus	None	Peritoneum	2
Polyoma virus	None	Subcutaneous	3
Polyoma virus	CaPO ₄	Liver/spleen	4
v-src oncogene	None	Subcutaneous	5
Hepatitis virus	None/CaPO ₄	Liver	6
<i>Plasmid reporter genes</i>			
Insulin	Liposome	Intravenous	7
pSV2-CAT, pRSV-CAT, hGH, insulin, PEPCK-CAT, HBsAg	CaPO ₄	Peritoneum	8
PEPCK-CAT	Liposome	Peritoneum	9
pBR-SV40	Liposome	Liver	10
pSV2-CAT, pALB-CAT	Polycationic glycoprotein	Liver	11,12
pRSV-luc	Lipofectin	Brain	13,14
pSV2-β-gal, pMLV-CAT	Lipofectin	Brain	15
pSV2-CAT	Lipofectin	Lung	16
pSV2-β-gal	Lipofectin	Artery	17
pRSV-luc, β-gal, CAT	None	Muscle	18
pRSV-β-gal	None	Heart	19
pRSV-CAT, pCMV-β-gal, MMTV-β-gal	Particle bombardment	Liver, muscle, intestine, skin, mammary tissue	20,21

The experiments contributed to a better understanding of the role of polyoma, Rous sarcoma and hepatitis virus genes in *in vivo* virus replication and oncogenesis. The observed effects were primarily attributed to the ability of the systems under study to be amplified by replication of an extremely rare, low frequency oncogenic or infectious event. Discussion on how the apparent low level of expression obtained could be used to therapeutic benefit was limited. However, the results do show that infectious and oncogenic DNA sequences can be taken up and expressed by cells following direct injection *in vivo*.

Three general approaches have been reported for obtaining expression from directly injected non-replicating plasmid DNA sequences. First, *in vivo* gene expression has been reported following direct injection of non-infectious, non-oncogenic plasmid DNAs encapsulated in liposomes, immunoliposomes and in a liposome/red blood cell membrane hybrid^{7,9,10}. Second, expression from a variety of calcium phosphate-precipitated gene sequences was reported following direct intraperitoneal injection into newborn rats⁸. Third, Wu^{11,12} showed that complexes between an asialoorosomucoid/polylysine conjugate and plasmid reporter genes can target expression exclusively to the liver, following intravenous administration.

Recent results

Cationic lipid mediated. At least ten citations showing direct *in vivo* gene transfer

have appeared within the past year, which taken together suggest that research activity in this area is likely to increase significantly. Much of this research used the cationic lipid reagent, lipofectin, to facilitate *in vivo* uptake and expression. Malone¹³ and Holt¹⁴ showed that chloramphenicol acetyl transferase (CAT) and luciferase activities could be recovered from *Xenopus* frog embryos following direct injection of the appropriate plasmid into neural tissue. Activity was seen to persist for more than a month. Ono¹⁵ showed similar results in mouse brain. Brigham¹⁶ reported that intravenous injection of complexes of lipofectin and CAT plasmid resulted in measurable CAT activity in the lungs of mice. And Nabel¹⁷, showed that plasmids can be lipofected into the arterial wall in the space between the two balloons of a double-balloon catheter.

Non-lipid mediated. Wolff and Felgner¹⁸ demonstrated reporter gene expression from plasmids containing luciferase, CAT and beta-galactosidase genes following direct injection into mouse skeletal muscle *in vivo*. Up to 1 ng of gene product could be isolated from tissues injected with 10–100 µg of plasmid DNA. Although there was no evidence of integration into the host genome, expression persisted for more than six months. Integration is not expected in the fully differentiated, non-dividing muscle fibres in which the expression was detected, as integration requires cell division. Persistent expression, therefore, implies that the gene sequences, upon reaching the nucleus, are main-

tained and repaired like normal DNA. *In vitro* transcribed messenger RNA is also taken up and expressed in mouse muscle, but the duration of expression is much shorter. Interestingly, no special delivery system is required for these effects. Similar results in cardiac muscle were subsequently reported by Leiden and colleagues¹⁹.

Microparticle bombardment. McCabe²⁰ and Johnston²¹ used high-energy micro-particle bombardment to introduce functional genes into living mammalian tissue, with devices such as the Biolistic device produced by Du Pont (Wilmington, Delaware). Submicron-sized tungsten or gold particles spontaneously adsorb DNA. These 'microprojectiles' can be accelerated to high velocity by various types of combustion procedures, including a gun powder discharge. Expression following bombardment was observed in skin, muscle, liver, intestine and mammary gland.

Applications

Immune regulation. Recent studies have demonstrated that there are two different pathways of antigen processing²²⁻²⁴. Proteins that are transported into a cell are processed by one pathway, activate helper T cells and, ultimately, stimulate the humoral immunity. Proteins synthesized within a cell are degraded by a separate process and stimulate cellular immunity. The ability to deliver functional nucleic acids to cells offers two new approaches to vaccination. First, the expression of secreted protein antigens may allow the production of subunit vaccines that stimulate both arms of the immune system. Second, intracellular expression of a non-secreted antigen should specifically induce or stimulate cellular immunity. Enhancing the cytotoxic response to a viral protein may allow the control of chronic or latent viral infec-

tions. Thus, in addition to disease prevention, vaccination could be used for the treatment of diseases.

Some of these concepts have been tested by using a plasmid containing the gene for a secreted form of the human immunodeficiency virus gp120 protein and driven by the cytomegalovirus immediate early promoter (in collaboration with N. Haigwood, Chiron Corporation). A single intramuscular injection of this plasmid induces a high titre of IgG antibodies (in preparation). The same injection scheme also induces cellular immunity to the protein (unpublished observation). An alternative vaccination strategy in which cationic lipids are used to transfected syngeneic cells has also been tested. It has been determined that a single injection of three million transiently transfected lymphoid or fibroblastic cells also induces a secondary humoral immune response (unpublished observation). These preliminary findings suggest that vaccines may be one of the first applications of direct gene delivery techniques.

Genetic disease. Perhaps the most obvious extension of the skeletal and heart muscle findings is in the management of muscular dystrophy. The full length dystrophin gene, which is defective in patients with muscular dystrophy, has recently been sequenced and efforts are underway in several laboratories to produce a plasmid containing the full length cDNA. Because of its large size and the complex intracellular network into which dystrophin is organized, it will be difficult to deliver functionally the recombinant dystrophin molecule. Intramuscular administration of the gene would, in principle, enable the cell to incorporate the dystrophin protein appropriately underneath the plasma membranes, making a gene therapy approach to this disease highly desirable.

Other genetic diseases that might be amenable to the direct approach may be: (1) cystic fibrosis, by aerosolization of the transmembrane regulatory gene to lung; (2) Parkinson's disease, by direct injection into the brain of the tyrosine hydroxylase gene; and (3) hypercholesterolaemia, by delivery of the high density lipoprotein receptor to liver with cationic orosomucoid complexes.

Therapeutic secreted proteins. The results with reporter genes that are reviewed here suggest that the local area of introduction of genes into tissues can serve as a depot for the synthesis, controlled release and secretion of therapeutic proteins. Initial candidates for this application are highly potent hormones and growth factors that can have beneficial effects at low concentrations (for example, growth hormone, erythropoietin and parathyroid hormone). In principle, a gene such as the insulin gene, could be

placed under the control of a regulatable promoter to allow release of the molecule in response to the metabolic status of the patient. Because of the blood/brain barrier, systemically administered recombinant growth factors are typically inaccessible to brain. Direct introduction of genes into brain tissue may provide a means of administering beneficial brain growth factors for the treatment of diseases of the central nervous system.

Summing up

Taken together, these findings identify a new paradigm for the therapeutic use of DNA and messenger RNA. Classical gene therapy strives for a one-time treatment leading to a permanent cure. The results reviewed here suggest that functional genes may be administered in a 'drug-like' manner, repetitively and on an 'as needed' basis. When additional gene product is required for therapeutic effect, more gene can be administered. When the treatment is no longer required, or if side effects develop, the treatment can be stopped. In order to distinguish this new paradigm from classical gene therapy, we have termed it 'gene therapeutics'. □

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Tools for gene technology

A kit to avoid false positives with PCR and YAC vectors will feature at next week's Miami Bio/Technology Winter Symposium on the Molecular Biology of Human Genetic Disease to be held in Miami, Florida.

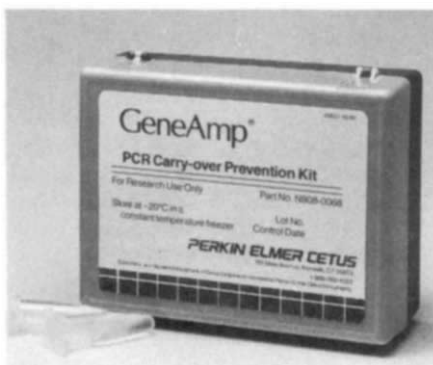
THE Poly(A) RNA Isolation Kit from United States Biochemical is designed to provide a quick and easy means of obtaining messenger RNA from total cellular RNA in under two hours (*Reader Service No. 101*). The kit uses oligo(dT)-cellulose spin columns to minimise the handling of RNA samples and maximise the flow rate of wash and elution buffers through the column, says USB. Typically, 2–5 per cent of the total starting RNA is isolated from the columns as mRNA, which is then suitable for use as a template for cDNA synthesis and the preparation of cDNA libraries. The \$165 (US) kit contains sufficient reagents for two complete mRNA isolations when starting with 1–5 mg of total RNA per isolation. USB will be in booths 16/17.

Free copies of the 1991 radiochemicals catalogue are now available from Sigma (*Reader Service No. 102*). Over 350 radiolabelled products and reagents are featured, including a line of cell culture-tested radiochemicals in convenient new multi-dose vials. Other new product releases include a range of LSC cocktails and the radioactivity decontaminant, Sigmacean. See Sigma's specialty biochemicals in booth 47.

GeneWorks is the new nucleic acid and protein sequence analysis software package from Intelligenetics that runs on the Apple Macintosh (*Reader Service No. 103*). The graphic environment of the GeneWorks program allows the user to view a sequence from several different perspectives simultaneously, perform analyses and manipulate linear sequence data as texts, maps, plots and graphs. Using the program's drawing and editing tools and various formatting options, users can produce publication-quality output. GeneWorks can read and write files in formats that are compatible with Intelligenetics' other programs and with EMBL, SWISS-PROT and GenBank. Intelligenetics will be in booth 1.

DNA details

Avoid false positives with PCR, says Perkin-Elmer Cetus, with the new GeneAmp PCR Carry-over Prevention Kit (*Reader Service No. 104*). By substituting dUTP for dTTP in the PCR reaction mix and by using uracil N-glycosylase (UNG) for the excision of uracil residues as a pretreatment step for



PCR carryover prevention kit.

subsequent PCR reactions, the kit can be used to ensure that DNA products from previous PCR amplifications cannot be reamplified to produce false positives. UNG is active on both single- and double-stranded DNA, and so the procedure can be applied to PCR products from standard, denatured, or asymmetric PCR amplifications. In addition, ribouracil residues in RNA and dUTP are not substrates for UNG, so the kit will not interfere with RNA PCR, says the company. The GeneAmp PCR Carry-over Prevention Kit contains sufficient reagents to perform 100 reactions, each of 100 μ l. See booths 55–57.

Molecular biologists needing to clone large DNA fragments of 100 to 1,000 kb in length, may be interested in the new vector system, YAC vectors pJS97/pJS98, developed by Life Technologies (*Reader Service No. 105*). pJS97 and pJS98 are plasmids, each of which constitutes one half of a YAC. To provide flexibility with cloning strategies, the plasmids have a multiple cloning site with seven restriction sites, including *EcoR* I, *Not* I and *Sal* I. In addition, each vector arm has a marker selectable in yeast: *URA* for pJS97 and *TRP* for pJS98. When ligated together with insert DNA, this allows double selection, says the company, thereby ensuring the selection of clones with both chromosome arms. Also, T7 RNA polymerase promoters facilitate chromosome mapping strategies. Life Technologies will be in booths 24/33.

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can be controlled by an IBM or compatible computer. In addition to the standard methods for 0.2-, 1.3- and 10- μ mol synthesis, the instrument includes a 'Custom Method' option. By modifying the valve positions and flow rates, existing methods can be tailored to novel research applications, says Pharmacia. Gene Assembler Plus is intended for the production of oligonucleotides for PCR, sequencing primers, hybridization probes, linkers, adaptors, as well as structural DNA studies and the creation of synthetic genes. Visit Pharmacia in booths 44-46.

Biotin-label oligonucleotides in a one-step process that uses biotin phosphor-

phoramidite, the new reagent from NEN Research Products that is available from Du Pont (*Reader Service No. 107*). This method of making 5'-biotinylated oligonucleotides uses a biotinylated phosphoramidite to link biotin directly to an oligonucleotide during the course of a typical automated oligonucleotide synthesis. In addition to their use as hybridization probes, the 5'-biotinylated oligonucleotides can, says the company, be used in ligase-mediated gene direction, direct dideoxy sequencing following PCR and in nonradiometric sequencing. Biotin phosphoramidite costs £230 (UK) for a five-vial pack. Each vial contains 28 mg, which is sufficient for one 5'-biotin labelling of an oligonucleotide at the 1- μ mol level. The reagent can be used with any DNA synthesizer. See Du Pont in booth 23.

DNA sequencing

Dynal will be launching its new Dynabeads Template Preparation Kit in Miami, which is designed for the **preparation of clean and reproducible templates for DNA sequencing** (*Reader Service No. 108*). The \$560 (US) kit produces a pure, single-stranded template for solid-phase DNA sequencing directly from a single colony in less than two hours using the streptavidin-biotin system, says Dynal. Universal biotinylated primers that can be used with common cloning vectors that are based on blue/white colour selection (for example, pUC, M13, pBluescript II or λ gt11) provide rapid *in vitro* amplification of the insert, while the Dynabeads M-280 product coated with streptavidin immobilizes the product. After melting, the pure, immobilized template is ready for standard dideoxy sequencing procedures. A rapid magnetic separation using the \$560 (US) Dynal MPC-96, a microtray separation device, completes the procedure. The procedure is optimal for large-scale sequencing and automation, says Dynal. See booth 4.

Using the SEQ-EASY Digitizer/Talker offered by DNASTAR, users can **enter sequence gel autoradiograms directly into an IBM PC/compatible or Macintosh computer** (*Reader Service No. 109*). The full-size light table can accommodate most types of gels. Users can choose between speech synthesis or musical tones for prompting or proofreading. Gels can be read from either the 3' or 5' end. According to DNASTAR, an inexperienced user can accurately input data from film to computer at 100 bases per minute; 200 bases per minute with practice. Input data can then be analysed with any of the 55 analysis functions offered by the company. See the full array of DNASTAR's products in booth 37.

SeqEd DNA sequence editing software from Applied Biosystems for use with the

company's fluorescence-based 373A DNA sequencing system is designed to provide an efficient method for sorting and analysing sequence data (*Reader Service No. 110*). The software allows the simultaneous display of multiple, related DNA sequences and the primary sequence data in the form of four-colour chromatograms. Individual base calls can be resolved using SeqEd and different alignment algorithms within the software can accommodate diverse DNA sequencing applications. Applied Biosystems' sequencing systems will be in on display in booths 18/38.

Probing matters

The molecular cytogenetics division of Oncor offers a complete product system for **chromosome analysis** using *in situ* hybridization techniques, with kits and biotin-labelled satellite DNA probes for all human chromosomes (*Reader Service No. 111*). Stated applications include chromosome identification in clinical cytogenetics, determination of ploidy, and detection of aneuploidy in tumour cells or in routine prenatal or postnatal chromosome preparations. A new product launch at the winter symposium will be Blockit. The kit consists of highly purified, sized human DNA and hybridization solution, and can be used for the suppression of repetitive DNA sequences in chromosome *in situ* hybridization with cosmid and YACs. New probes for cancer research will also be on display, including oestrogen receptor, GST π , p53, *c-erb B-2*, TGF α , TGF β , *mdr 1*, E2A, vimentin and tubulin. Oncor's wares will be on show in booth 7.

The new TAG-IT Labelling Kit from Bios **simultaneously amplifies DNA as it labels** (*Reader Service No. 112*). The kit synthesizes as much as thirteen times the amount of starting template while incorporating radiolabelled deoxyribonucleotides to a specific activity of 0.75 (± 0.05) $\times 10^9$ d.p.m. μ g $^{-1}$, says Bios. The procedure can be initiated with as little as 1 ng of DNA. The TAG-IT Kit can be used with templates of any size and does not require template sequence information. The procedure uses *Taq* polymerase and thermal cycling. Use of *Taq* polymerase enables longer probes to be generated than with conventional methods; this permits better coverage of target DNA immobilized on filters and the formation of a more stable probe-target duplex, says Bios. Stop by the Bios booth, number 22. $\square$

These notes are compiled by Diane Gershon from information provided by the manufacturers. To obtain further details, use the reader service card bound inside the journal. Prices quoted are sometimes nominal, and apply only within the country indicated.

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